In-vitro-Studien zur Charakterisierung der biologischen Funktion langkettiger Vitamin-E-Metaboliten

Dissertation
zur Erlangung des akademischen Grades
„doctor rerum naturalium“ (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät der
Friedrich-Schiller-Universität Jena

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geboren am 24.10.1987 in Nürnberg

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Tag der Verteidigung

15. Mai 2018

Vorwort

Obwohl Vitamin E schon 1922 durch EVANS & BISHOP entdeckt wurde, ist seine Funktion im menschlichen Körper bis heute noch nicht vollständig aufgeklärt. Die Gründe hierfür sind vielfältig, aber sicherlich wichtig ist die inzwischen erfolgende Verschiebung des Forschungsinteresses auf unterschiedliche Funktionen des Vitamins.

Zu Beginn standen die Auswirkungen eines Vitamin-E-Mangels im Mittelpunkt des wissenschaftlichen Interesses. Eine Vitamin-E-defiziente Ernährung führte bei Ratten zur Einschränkung ihrer Reproduktionsfähigkeit (EVANS & BISHOP 1922). Im Menschen hingegen manifestiert sich ein Mangel an Vitamin E nur in wenigen Fällen ernährungsbedingt (siehe Kapitel 1.4).

Daher konzentriert sich die Forschung seitdem vermehrt auf die Aufklärung der spezifischen zellulären Vitamin-E-Funktionen (siehe Kapitel 1.8) und deren Einfluss auf die Prävention und Pathogenese vielfältiger Krankheiten, z.B. der kardiovaskulären Erkrankungen (Manuskript 5). Zahlreiche, vielversprechende *In-vitro*-Untersuchungen zur antiatherogenen Wirkung des Vitamins führten zu einer vermehrten Durchführung großer Humaninterventionsstudien, welche durch uneinheitliche oder teils auch negative Ergebnisse die Euphorie dämpften (Manuskript 5). Dem folgten eine Ernüchterung und dadurch eine Verlangsamung in der Aufklärung der zugrundeliegenden Mechanismen.

Parallel zu dieser Entwicklung wurde in den 1980er Jahren des vergangenen Jahrhunderts der Abbau des Vitamin E in der Leber beschrieben, welcher eine Intoxikation mit demselben unterbindet (Kapitel 1.9, Manuskript 6; TRABER 2013). Durch diese Erkenntnisse konnten neue Biomarker für die Vitamin-E-Versorgung etabliert werden (LEBOLD *et al.* 2012). Die biologische Funktion der kurzkettigen Metabolite stand dabei schon frühzeitig im Fokus des Interesses.

Erst Anfang dieses Jahrtausends erweiterte sich das Forschungsgebiet um die zu diesem Zeitpunkt erstmalig beschriebenen langkettigen Metabolite (SONTAG & PARKER 2002). Seither arbeiten eine internationale Forschergruppe (Deutschland, Italien, Australien und Frankreich um Stefan Lorkowski (Institut für Ernährungswissenschaften, Friedrich-Schiller-Universität Jena) und Marc Birringer (Angewandte Biochemie für Ernährung und Umwelt, Hochschule Fulda)) und eine Gruppe in den USA um Qing Jiang an der Aufklärung ihrer biologischen Funktion. Obwohl das Wissen auf diesem Gebiet noch lückenhaft ist, wurde dieses Jahr in einem Konsensusartikel zum 2. Internationalen Vitamin-E-Satellitensymposium bereits die Bedeutung der langkettigen Metabolite herausgestellt (GALLI *et al.* 2017).

Die vorliegende Arbeit entstand im Rahmen der Aktivitäten der oben genannten internationalen Forschergruppe und strebt die Aufklärung der biologischen Funktion der langkettigen Vitamin-E-Metabolite an. Dieses Ziel ist innerhalb einer Dissertation alleine nicht zu erreichen; daher setzt sie die Forschungsarbeit von WALLERT (2014) fort und legt die Basis für weiterführende Arbeiten.

Die oben erwähnte Diskrepanz der *In-vitro*- und *In-vivo*-Studien im Kontext der kardiovaskulären Erkrankungen führte zu folgender Überlegung: Könnte der Metabolismus von Vitamin E, der *in vivo* im Gegensatz zu *in vitro* (im Abhängigkeit von der eingesetzten Zelllinie) stattfindet, für den Ausgang der Studien verantwortlich sein? Aus diesem Anlass fokussiert sich die vorliegende

Arbeit auf Prozesse, die in der Entstehung der Atherosklerose eine wichtige Rolle spielen, nämlich die Inflammation und die zelluläre Lipidhomöostase. Die Manuskripte 1 bis 4 beschreiben dabei die biologischen Effekte der langkettigen Vitamin-E-Metabolite in diesem Kontext, wobei das Manuskript 1 darüber hinaus eine enge Strukturabhängigkeit der Effekte aufzeigt. Diese Ergebnisse deuten auf die Existenz eines spezifischen Rezeptors für die langkettigen Vitamin-E-Metabolite hin.

Die internationale Forschergruppe steckt sich das ehrgeizige Ziel die Funktionsweise des Vitamin E bis zu seinem hundertsten Jubiläum im Jahr 2022 aufzuklären. Dies scheint durch den Nachweis der biologischen Aktivität der langkettigen Metabolite noch ambitionierter. Aber gerade die sich mit dieser Arbeit andeutende Homologie von Vitamin E zu anderen fettlöslichen Vitaminen, wie A und D, ist wissenschaftlich besonders reizvoll: die biologisch aktive Form von Vitamin A und D ist jeweils ein physiologisches Abbauprodukt, für das ein spezifischer Rezeptor bereits identifiziert wurde. Sollte diese Hypothese nun auch für Vitamin E bestätigt werden, würde dies zu einem weiteren Umdenken in der Vitamin-E-Forschung führen und das Konzept der Bioaktivierung fettlöslicher Vitamine allgemeingültiger machen.

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11'S-COOH	11'-Carboxychromanolsulfat (analog für Metabolite anderer Kettenlänge)
13'-COOH	13'-Carboxychromanol (analog für Metabolite anderer Kettenlänge)
13'-OH	13'-Hydroxychromanol
3'-COOH	Carboxyhydroxyethylchromanol
5-LO	5-Lipoxygenase
ABCA1	*ATP-binding cassette transporter A1*
ADRP	Adipophilin, *adipose differentiation related protein*
APCI	*atmospheric pressure chemical ionization*
ATP	Adenosintriphosphat
AVED	*ataxia with vitamin E deficiency*
CD36	*cluster of differentiation 36*
$CDMD(en)_2HC$	Carboxydimethyldecadienylhydroxychromanol
CDMDHC	Carboxymethyldecahydroxychromanol
CDMHenHC	Carboxymethylhexenylhydroxychromanol
CDMHHC	Carboxymethylhexylhydroxychromanol
$CDMO(en)_2HC$	Carboxydimethyloctdienylhydroxychromanol
CDMOHC	Carboxymethyloctylhydroxychromanol
CEHC	Carboxyhydroxyethylchromanol
CEHC	Carboxyethylhydroxychromanol
CETP	Cholesterolestertransferprotein
CKD	chronisches Nierenversagen, *chronic kidney disease*
CMBenHC	Carboxymethylbutadienylhydroxychromanol
CMBHC	Carboxymethylbutylhydroxychromanol
COX	Cyclooxygenase
CR	Chylomikronenüberbleibsel, *chylomicron remnants*
CYP	Cytochrom P450
CYP3A4	Cytochrome-P450-abhängige Enzyme der Familie 3A4
CYP4F2	Cytochrome-P450-abhängige Enzyme der Familie 4F2
DGE	Deutsche Gesellschaft für Ernährung

DMSO	Dimethylsulfoxid
ECD	*electron capture detector*
EERM	von epigenetischen Enzymen benötigte Metabolite, *epigenetic enzyme required metabolites*
EFSA	Europäische Behörde für Lebensmittelsicherheit, *European Food Safety Authority*
ELISA	*enzyme linked immunosorbent assay*
EMSA	*electrophoretic mobility shift assay*
ESI (+) oder ESI (-)	Elektronensprayionisation im positiven oder negativen Modus
FATP	Fettsäuretransportprotein, *fatty acid transport protein*
FD	Fluoreszenzdetektion
GA	Garciniasäure, *garcinoic acid*
GC	Gaschromatographie
HDL	Lipoproteine hoher Dichte, *high density lipoproteins*
HPLC	Hochdruckflüssigchromatografie, *high pressure liquid chromatography*
IC_{50}	mittlere inhibitorische Konzentration
ICM	Mittelkettiger Vitamin-E-Metabolit, *intermediate-chain metabolites*
IDL	Lipoproteine mittlerer Dichte, *intermediate density lipoprotein*
IHME	*Institute for Health Metrics and Evaluation*
iNOS	induzierbare Stickstoffmonoxidsynthase, *inducible nitric oxide synthase*
LC	Flüssigchromatografie, *liquid chromatography*
LCM	langkettige Vitamin-E-Metaboliten, *long-chain metabolite*
LDL	Lipoproteine geringer Dichte, *low density lipoprotein*
LDLR	LDL-Rezeptor
LPL	Lipoproteinlipase
LPS	Lipopolysaccharid
LRP	LDL-Rezeptor assoziiertes Protein, *LDL receptor related protein*
LT	Leukotrien

LTB_4	Leukotrien B_4
MDR3	*multidrug resistance protein 3*
mPGES-1	mikrosomale PGE2-Synthase-1
mRNA	Boten-RNA, *messenger RNA*
MS	Massenspektrometrie
NFκB	*nuclear factor kappa-light-chain-enhancer of activated B-cells*
NPC1L1	*Niemann-Pick C1-like 1*
Nrf2	*nuclear factor 2*
oxLDL	oxidiertes LDL
PARP-1	Poly-ADP Ribose Polymerase-1
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
P-gp	P-Glykoprotein
PMNL	polymorphkernige neutrophile Leukozyten
PLIN2	Adipophilin, Perilipin 2 *adipose differentiation related protein*
PLTP	Phospholipidtransferprotein
PMA	Phorbol-12-Myristat-13-Acetat
PPARγ	*peroxisome proliferator-activted receptor γ*
PXR	*pregnane X receptor*
RDA	empfohlene Tagesdosis, *recommended daily allowance*
RNA	Ribonukleinsäure, *ribonuclelic acid*
ROS	reaktive Sauerstoffspezies, *reactive oxygen species*
RT-qPCR	quantitative Reverse-Transkriptase-Polymerasekettenreaktion, *quantitative reverse transcriptase polymerase chain reaction*
SA	Stearinsäure
SCD	Stearoyl-Coenzym-A-Desaturase
SCM	kurzkettiger Vitamin-E-Metabolit, *short-chain metabolites*
siRNA	*short interfering RNA*
SMase	Sphingomyelinasen
SPE	*solid phase extraction*

SR-BI	*scavenger receptor-B1*
T3	Tocotrienol
TE	TOH-Äquivalent, *tocopherol equivalent*
TOF	*time of flight*
TOH	Tocopherol
UV	Detektor für Ultraviolettstrahlung
VLDL	Lipoproteine sehr geringer Dichte, *very low densitiy lipoproteins*
α-TTP	α-Tocopherol-Transferprotein

1 Vitamin E

Charakteristisch für die Struktur des Vitamin E sind der Chromanolring und die isoprenoide Seitenkette. Das Ringsystem ist mit Methylgruppen unterschiedlicher Anzahl an unterschiedlichen Positionen substituiert und wird als α-, β-, γ- oder δ-Form eindeutig beschrieben. Die Sättigung der Seitenkette entscheidet über die Bezeichnung als Tocopherol (TOH, gesättigte Seitenkette) oder Tocotrienol (T3, ungesättigte Seitenkette). Abbildung 1 gibt einen Überblick über die acht bekannten Vitamin-E-Formen. Da die chemische Struktur, hierbei insbesondere die isoprenoide Seitenkette, eine geringe Wasserlöslichkeit des Vitamins bedingt, zählt Vitamin E zu den fettlöslichen Vitaminen.

Außerdem finden sich in der Natur vielfältige, dem Vitamin E ähnliche Verbindungen. Diese verfügen alle über ein Chromanolringsystem, variieren aber in der Länge der Seitenkette. Bei einer Strukturrecherche konnten etwa 170 dem Vitamin E ähnliche Verbindungen identifiziert werden (persönliche Mitteilung von Marc Birringer, Hochschule Fulda; Manuskript in Vorbereitung).

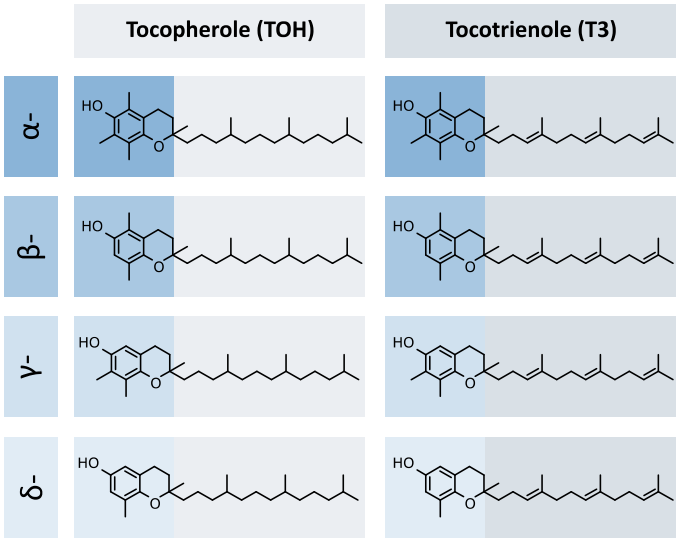


Abbildung 1: Vitamin-E-Formen.
Vitamin E als Sammelbegriff für Tocopherole und Tocotrienole (Unterschiede in der Sättigung der Seitenkette) in α-, β-, γ- oder δ-Formen (Unterschiede in der Substitution des Ringsystems).

1.1 Vorkommen und Stabilität

Aufgrund seiner hohen Fettlöslichkeit findet sich Vitamin E vorranging in Lebensmitteln mit hohem Fettanteil. So sind insbesondere pflanzliche Speiseöle, wie Weizenkeimöl (151 - 192 mg α-TOH/100 g) und Sonnenblumenöl (33 - 59 mg α-TOH/100 g) oder auch Sojaöl (61 - 70 mg γ-TOH/100 g) und Maisöl (44 - 75 mg γ-TOH/100 g) gute Quellen für α- oder γ-TOH (SHAHIDI & CAMARGO 2016).

Die Stabilität von Vitamin E in Lebensmitteln ist vergleichsweise gut, hängt aber von den Lagerbedingungen, der Verarbeitung der Lebensmittel und der Matrix ab (CHAPMAN *et al.* 2009). So unterliegt Vitamin E unter Einwirkung von Licht und Luftsauerstoff schnell der Oxidation

(PIGNITTER *et al.* 2014) und ist unter Frittierbedingungen nicht stabil (RÉBLOVÁ 2006). Auch die Fettsäurezusammensetzung der Pflanzenöle spielt für die Stabilität eine entscheidende Rolle, denn Vitamin E oxidiert in Ölen mit hohem Anteil an ungesättigten Fettsäuren schneller (VERLEYEN *et al.* 2002).

1.2 Verzehr

Der Verzehr von Vitamin E liegt weltweit im Mittel bei 10,2 mg Vitamin E/d (Median) und variiert stark (1,1 - 134,2 mg Vitamin E/d; PÉTER *et al.* 2016). In Deutschland wird nach Angaben der Nationalen Verzehrsstudie II im Median eine Zufuhr von 12 mg Vitamin E/d bei Frauen und 14 mg Vitamin E/d bei Männern erreicht (MRI 2008). In Abhängigkeit von der bevorzugten Lebensmittelquelle ist der anteilige Verzehr der Vitamin-E-Formen regional verschieden. Während in Europa mehr α-TOH-reiche Lebensmittel verzehrt werden, ist der Anteil des γ-TOH (etwa 70 % des zugeführten Vitamin E) durch den größeren Verzehr von z.B. Mais und Soja in den USA höher (JIANG *et al.* 2001).

1.3 Empfehlungen

Die Deutsche Gesellschaft für Ernährung (DGE e.V.) empfiehlt Erwachsenen eine Aufnahme von 12 bis 15 mg TOH-Äquivalente (*tocopherol equivalent*, TE) pro Tag (DGE 2013). Bei einer gemischten Zufuhr an Tocopherolen kann die benötigte Dosis aus den in der Tabelle 1 dargestellten Mengenäquivalenten bestimmt werden. Zur Festlegung dieser Werte dient die Bestimmung der relativen biologischen Aktivität im Fötusresorptionstest in Ratten (KAMAL-ELDIN & APPELQVIST 1996).

Tabelle 1: Gegenüberstellung der biologischen Aktivität der Tocopherole und ihre Übertragung in TOH-Äquivalente nach KAMAL-ELDIN & APPELQVIST (1996).

	Aktivität im Resorptionstest	Menge in mg für 1 TE
RRR-α-TOH	100 %	1
RRR-β-TOH	50 %	2
RRR-γ-TOH	10 %	10
RRR-δ-TOH	3 %	33,3
RRR-α-T3	30 %	3,3

Verwendete Abkürzungen: TE, *tocopherol equivalent;* TOH, Tocopherol; T3, Tocotrienol.

Die DGE legt den empfohlenen Referenzwerten den Vitamin-E-vermittelten Schutz vor Lipidperoxidation zugrunde (DGE 2013). Durch einen vermehrten Verzehr von ungesättigten Fettsäuren steigt das Risiko der Lipidperoxidation, weshalb sich der Bedarf an Vitamin E in diesem Fall erhöht (0,5 mg TE/g mehrfach ungesättigter Fettsäuren; RAEDERSTORFF *et al.* 2015). In den USA hingegen wird die empfohlene Tagesdosis (*recommended daily allowance*, RDA) für Erwachsene

mit 15 mg/d angegeben und basiert auf Werten, die zur Vermeidung einer peroxidinduzierten Hämolyse in Blutproben von Vitamin-E-defizienten Personen experimentell nötig waren (PÉTER *et al.* 2016). Diese Herangehensweise wird in den USA als Goldstandard angesehen, ist aber nicht unumstritten (AZZI 2017). Die Europäische Behörde für Lebensmittelsicherheit (*European Food Safety Authority*, EFSA) gibt eine sichere, maximale Tageszufuhr von 300 mg/d an (EFSA 2006).

1.4 Status und Mangel

Beim Vergleich der tatsächlichen Zufuhr an Vitamin E mit den in den Referenzwerten angegebenen Zufuhrempfehlungen wird eine Diskrepanz deutlich. Weltweit erreichen 82 % der Menschen die in den USA empfohlene Tagesdosis nicht (PÉTER *et al.* 2016); in Deutschland werden die DGE-Empfehlungen von etwa 50 % der Erwachsenen nicht eingehalten (MRI 2008). Da in den meisten Fällen eine moderate Unterversorgung aber symptomlos bleibt, regten einige Wissenschaftler eine Überarbeitung der scheinbar zu hohen Referenzwerte an (NOVOTNY *et al.* 2012).

Basis für die Festlegung der neuen Empfehlungen könnten z.B. Vitamin-E-Blutspiegel sein, die einen Hinweis auf den Vitamin-E-Status liefern könnten. Der Grenzwert für den Vitamin-E-Mangel wird hierbei vom Auftreten negativer Effekte abhängig gemacht. So spricht man ab einem α-TOH-Serumspiegel von < 8 µmol/l α-TOH von einem manifesten Vitamin-E-Mangel, während bei < 12 µmol/l α-TOH im Serum funktionelle Einschränkungen erwartet werden (PÉTER *et al.* 2016). Mangelsymptome sind Ataxie (*ataxia with vitamin E deficiency*, AVED; SCHUELKE 2005) oder auch kognitive Dysfunktion (FUKUI *et al.* 2015) sowie negative Effekte auf die Reproduktionsfähigkeit (LEBOLD & TRABER 2014), wie sie tierexperimentell zur Entdeckung der Vitamin-E-Wirkung führten (Fötus-Resorption durch Vitamin-E-Mangel bei Ratten; EVANS & BISHOP 1922). Einige Studien legen nahe, dass ein α-TOH-Serumspiegel von > 30 µmol/l zu positiven Effekten bei der Prävention kardiovaskulärer Erkrankungen und unterschiedlicher Krebsarten führen könnte (PÉTER *et al.* 2016). Daher legt die DGE den Zielwert für die α-TOH-Serumkonzentration auf 30 µmol/l fest (DGE 2013). Weltweit liegt der unadjustierte Median der α-TOH-Serumkonzentration bei 22,1 µmol/l (PÉTER *et al.* 2016).

Manche Studien beziehen zur Beurteilung des Vitamin-E-Status die Vitamin-E-Konzentration im Serum oder Plasma auf die Gesamtcholesterolkonzentration (Vitamin E [µmol/mmol]; FORD *et al.* 2006b). Dies basiert auf einer engen Korrelation der beiden Messgrößen, birgt aber Risiken, da in Abhängigkeit von der Gesamtcholesterolkonzentration und dem Gesundheitszustand der Probanden der Vitamin-E-Status entweder richtig abgebildet, über- oder unterschätzt wird (VERES *et al.* 2017). Daher wird vorgeschlagen möglichst alle Informationen (Vitamin-E-Konzentration, Gesamtcholesterolkonzentration und Vitamin E je mmol Gesamtcholesterol) zur Verfügung zu stellen, damit der Einfluss der jeweiligen Messgrößen deutlich wird (VERES *et al.* 2017). Ein Beispiel aus der Studie von FORD *et al.* (2006b) verdeutlicht das Problem: ein 53-jähriger Mann mit einer normalen Vitamin-E-Konzentration von 18,4 µmol/l, aber einer sehr hohen Gesamtcholesterolkonzentration von 8,6 mmol/l hat ein Vitamin-E-Gesamtcholesterolverhältnis von 2,14 µmol/mmol, welches nach der studieneigenen Definition (< 2,22 µmol/mmol) sehr

niedrig ist. Wäre nur das Verhältnis aus Vitamin E und Gesamtcholesterol angegeben, könnten keine Rückschlüsse gezogen werden, ob die Vitamin-E-Konzentration zu niedrig oder die Gesamtcholesterolkonzentration zu hoch ist.

Genetische Faktoren spielen bei der Entstehung eines Vitamin-E-Mangels eine Rolle, wenn Mutationen in Genen auftreten, die für den Vitamin-E- oder Lipidstoffwechsel funktionell relevant sind. So können Mutationen z.B. im α-Tocopherol-Transferprotein (α-TTP; BROMLEY *et al.* 2013; siehe Kapitel 1.6.2), die Stoffwechselerkrankung zystische Fibrose (aufgrund einer Einschränkung in der Sekretion von z.B. Gallensäuren; RANA *et al.* 2014) oder Störungen des Lipoproteinstoffwechsels (z.B. familiäre Hypocholesterolämien, Abetalipoproteinämie, Hypobetalipoproteinämie oder die Chylomikronenretentionskrankheit; CUERQ *et al.* 2016; PERETTI *et al.* 2010), zu einem klinischen Mangel führen. Auch erworbene Komplikationen, wie z.B. Magenresektion (RINO *et al.* 2014) oder eine ausgeprägte Mangelernährung (PÉTER *et al.* 2013) können einen Vitamin-E-Mangel nach sich ziehen.

1.5 Bioverfügbarkeit

Die Bioverfügbarkeit von Vitamin E wird von vielfältigen Faktoren beeinflusst, die mit dem Akronym SLAMENGHI zusammengefasst werden (BOREL *et al.* 2013). Die Form des Vitamin E (*species*), die molekulare Verknüpfung (*linkage*, z.B. Veresterung), die verzehrte Menge (*amount*), die Lebensmittelmatrix (*matrix*), die Effektoren der Absorption und der Biokonversion (*effectors*, z.B. Lipide oder Ballaststoffe), der Status des Betroffenen (*nutritient status*), die genetischen Faktoren (*genetic factors*), sowie Charakterisika des Betroffenen (*host-related factors*, z.B. Geschlecht und Alter) und mathematisch berrechnete Interaktionen (*interactions*, z.B. zur Unterscheidung von Einzel- und Kombinationseffekten) können Einfluss nehmen.

1.6 Stoffwechsel und Transport von Vitamin E

1.6.1 Absorption

Nach dem Verzehr Vitamin-E-haltiger Lebensmittel erfolgt die Resorption zusammen mit Lipiden (siehe Abbildung 2). Im Magen setzt Pepsin Vitamin E aus der Lebensmittelmatrix frei (BOREL *et al.* 2013) und eine Emulgierung der Fettphase wird initiiert (ARMAND *et al.* 1996). Im Dünndarm erfolgt mit Hilfe von Gallensäuren eine weitere Emulgierung und anschließend die Resorption der Moleküle, meist rezeptorvermittelt, an der Oberfläche der Enterozyten (ABUMRAD & DAVIDSON 2012; ARMAND *et al.* 1996). Untersuchungen von BARDOWELL *et al.* (2012) legen nahe, dass bereits im Dünndarm eine Verstoffwechslung von Vitamin E stattfindet (siehe Manuskript 6). Die Aufnahme von α-TOH in Enterozyten wird unter anderem durch den Cholesteroltransporter *Niemann-Pick C1-like 1* (NPC1L1) vermittelt, wobei dessen N-terminale Domäne eine essentielle Rolle zu spielen scheint (KAMISHIKIRYO *et al.* 2017). Neben der intrazellulären Speicherung der Lipide in Lipidtropfen, werden diese über Chylomikronen in die Lymphe abgegeben (HUSSAIN 2014). Über die Pfortader und die anschließende Zirkulation im Blut gelangen die Lipide nach einem teilweisen Abbau über Lipasen in Form von Chylomikronenüberbleibseln (*chylomicron remnants*, CR) zur Leber und werden dort in die Leberzellen aufgenommen (JULVE *et al.* 2016).

Alternativ wird Vitamin E von den Enterozyten auch auf extrazelluläres Apolipoprotein A-I übertragen, welches dann durch eine weitere Beladung mit Lipiden zum Lipoprotein hoher Dichte (*high density lipoprotein*, HDL) wird (GREBENSTEIN *et al.* 2014; NIESOR 2015).

1.6.2 Vitamin-E-Stoffwechsel in der Leber

Die Leber ist für den Stoffwechsel von Vitamin E ein Organ von zentraler Bedeutung. Sie ist der Ort der Favorisierung bestimmter Vitamin-E-Formen und seines Metabolismus (Manuskript 6, Kapitel 1.6.2 und 1.9). Während über die Nahrung die unterschiedlichen Vitamin-E-Formen in variierenden Mengen zugeführt werden (Kapitel 1.2), ist α-TOH die vorherrschende Form im Körper. Das Verhältnis von α-TOH zu γ-TOH, die Vitamin-E-Form mit der zweithöchsten Konzentration im Blut, wird dabei mit 5,2 : 1 (FORD *et al.* 2006a) bis 41,2 : 1 (ZERBINATI *et al.* 2015) angegeben.

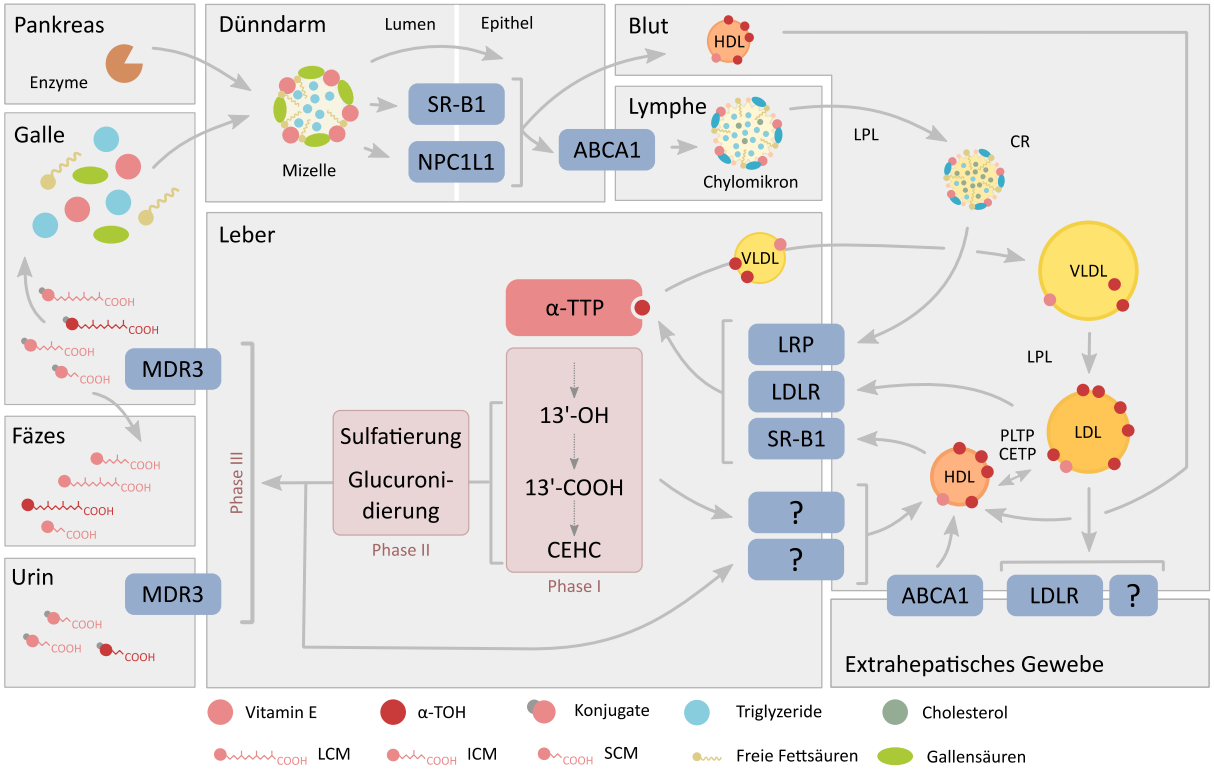


Abbildung 2: Vitamin-E-Resorption und -Stoffwechsel (siehe Text für Erläuterungen).
Verwendete Abkürzungen: 13'-OH, 13'-Hydroxychromanol; 13'-COOH, 13'-Carboxychromanol; α-TTP, α-Tocopherol-Transferprotein; ABCA1, *Adenosine triphosphate-binding cassatte transporter 1*; CETP, Cholesterolestertransferprotein; CR, *chylomicron remnants*; HDL, *high density lipoprotein*; LDL, *low densitiy lipoprotein*; LDLR, LDL-Rezeptor; LPL, Lipoproteinlipase; LRP, *LDL receptor related protein*; MDR3, *multidrug resistance protein 3*; ; NPC1L1, *Niemann-Pick C1 like 1*; PLTP, Phopholipidtransferprotein; SR-BI, *scavenger receptor-B1*; VLDL, *very low density lipoprotein*. Abbildung modifiziert nach Manuskript 6.

Dafür wird unter anderem das spezifische hepatische Protein α-TTP verantwortlich gemacht. Dieses weist unterschiedliche Bindungsaffinitäten zu den jeweiligen Vitamin-E-Formen auf (α-TOH: 100 %, β-TOH: 38 %, γ-TOH: 9 %, δ-TOH: 2 %, α-T3: 12 %; HOSOMI *et al.* 1997), schützt auf diesem Weg α-TOH vor dem Abbau und favorisiert daher dessen Zirkulation. Die intrazellulären Mechanismen werden in Manuskript 6 ausführlich beschrieben. Es gibt allerdings Hinweise, dass nicht nur die α-TTP-Bindungsaffinitäten für die α-TOH-Favorisierung eine Rolle

spielen, sondern ein Zusammenspiel aus α-TTP und Metabolismus dafür verantwortlich ist (GREBENSTEIN *et al.* 2014). Außerdem ist es denkbar, dass die anderen Vitamin-E-Formen unter engerer metabolischer Kontrolle stehen als α-TOH, da sie eine höhere Toxizität aufweisen (PARKER *et al.* 2004). Auf welchem Weg und aus welchem Grund α-TOH favorisiert wird, ist noch im Detail zu klären. Unumstritten ist allerdings, dass α-TOH die höchste biologische Aktivität besitzt (vgl. Ergebnisse des Fötusresorptionstest, Kapitel 1.2).

Nach der Verpackung des α-TOH und anderer lipophiler Moleküle in Lipoproteine sehr geringer Dichte (*very low densitiy lipoproteins*, VLDL) werden diese aus der Leber in die Zirkulation sekretiert und gelangen über den bekannten Lipoproteinstoffwechselweg (VLDL - IDL (*intermediate density lipoprotein*) - LDL (*low density lipoprotein*)) zum extrahepatischen Gewebe (HACQUEBARD & CARPENTIER 2005). Für α-TOH werden Bindeproteine beschrieben, die das Molekül intra- und extrazellulär transportieren (im Überblick in Manuskript 6).

1.7 Speicherung, zelluläre Verteilung und Orientierung in der Membran

In Ratten finden sich etwa 90 % des gesamten α-TOH im Skelettmuskel (42 %), in der Leber (28 %) und im Fettgewebe (21 %; BJØRNEBOE *et al.* 1990). Während die Leber als Kurzzeitspeicher für α-TOH dient, ist das Fettgewebe der Langzeitspeicher (MACHLIN & GABRIEL 1982), der z.B. durch Vitamin-E-Defizienz über vier Wochen bei Ratten nicht signifikant beeinflusst wird (UCHIDA *et al.* 2012).

Seiner lipophilen Natur folgend ist Vitamin E *in vitro* intrazellulär in membran- und lipidreichen Fraktionen zu finden, z.B. vorrangig in der Mikrosomenfraktion in Jurkat-Zellen (SAITO *et al.* 2004) oder in den Lipidtropfen in Fettzellen (TRABER & KAYDEN 1987). Der TOH-Gehalt in den zellulären Fraktionen verhält sich dabei proportional zum Lipidgehalt (SAITO *et al.* 2004).

Die Orientierung von α-TOH innerhalb der Membranen ist noch nicht eindeutig geklärt. So wird eine Ausrichtung parallel zur wässrigen Phase im Bereich der Phospholipidkopfgruppen (MARQUARDT *et al.* 2015) sowie eine Ausrichtung parallel zu den Fettsäureresten der Lipide (AUSILI *et al.* 2017) postuliert. Ebenfalls zur Diskussion steht, ob die Membranbeschaffenheit einen Einfluss auf die Verteilung des Vitamin E in der Membran hat (AUSILI *et al.* 2017; SÁNCHEZ-MIGALLÓN *et al.* 1996). Umgekehrt scheint allerdings Vitamin E die Membranbeschaffenheit zu beeinflussen: so bilden sich in oxidativ geschädigten Membranen Poren, welche durch α-TOH vermindert werden können (BOONNOY *et al.* 2017).

1.8 Funktionen

Erste Anhaltspunkte für die Funktion von Vitamin E lieferten die Symptome, welche unter Mangelbedingungen offensichtlich werden. So wurde Vitamin E aufgrund von Fertilitätsstörungen bei Vitamin-E-mangelernährten Ratten entdeckt (EVANS & BISHOP 1922). Auf molekularer Ebene übt Vitamin E antioxidative und von der antioxidativen Funktion unabhängige Effekte aus. Während die antioxidative Kapazität von Vitamin E schon lange bekannt ist (erste Erwähnung in PubMed im Jahr 1945, TOMARELLI & GYORGY) und als Grundlage für die Festlegung der jeweiligen Referenzwerte für die Zufuhr dient (siehe Kapitel 1.3), wurden die nicht-antioxidativen Funktionen

erstmals 1974 beschrieben (CATIGNANI *et al.*).

Der antioxidative Mechanismus ist sehr gut dokumentiert: Vitamin E dient als Fänger für Radikal, die z.B. bei der Lipidperoxidation von ungesättigten Fettsäuren durch Sauerstoff- und Lichteinwirkung entstehen (NIKI 2014). Daher wird es in der Lebensmittelindustrie gerne als Antioxidans eingesetzt (WITTING 1975). Auch *in vivo* bietet Vitamin E Schutz vor oxidativem Stress, wobei manche Forscher die Vitamin-E-Funktion auf diesen Aspekt reduzieren (TRABER & ATKINSON 2007). Die nicht-antioxidative bzw. genregulative Funktion von Vitamin E ist ebenfalls gut dokumentiert und wurde erst kürzlich zusammenfassend dargestellt (AZZI 2017; Manuskript 7).

1.9 Hepatischer Metabolismus

Zwei unterschiedliche Herangehensweisen ermöglichten die Aufklärung des Vitamin-E-Metabolismus. Zum einen lieferten *In-vitro*-Studien, z.B. mit Leberzellen, erste Hinweise auf entstehende Abbauprodukte (BIRRINGER *et al.* 2001; BIRRINGER *et al.* 2002; SONTAG & PARKER 2002). Zum anderen ergaben sich durch *In-vivo*-Studien mit markiertem (deuteriertem) TOH Hinweise auf den Metabolismus im Menschen (LEONARD *et al.* 2005; LODGE *et al.* 2001; TORQUATO *et al.* 2016b).

Der Abbau verläuft prinzipiell für alle Vitamin-E-Formen ähnlich. Die Effektivität des Metabolismus hängt von (mindestens) drei Faktoren ab: von der Substitution des Chromanolrings, der Sättigung der Seitenkette und der Quelle des Vitamins (Manuskript 6). Mit steigender Anzahl der Methylgruppen am Chromanolring sinkt die Abbaurate. Die TOH werden aufgrund ihrer gesättigten Seitenkette weniger abgebaut als die T3 und der Metabolismus von synthetischen Vitamin-E-Formen ist besonders hoch. So weist also das natürliche α-TOH die niedrigste Abbaurate auf (SONTAG & PARKER 2007). Der Metabolismus wird im Manuskript 6 sehr detailliert beschrieben und soll daher hier nur im Überblick anhand von α-TOH dargestellt werden (siehe Abbildung 3). Die ω-Hydroxylierung der Seitenkette wird durch Cytochrom-P450-abhängige (CYP) Enzyme katalysiert und erzeugt den ersten langkettigen Metabolit (*long-chain metabolite*, LCM), das α-13'-Hydroxychromanol (α-13'-OH). Die Frage, welche Enzymisoform für den Abbau des Vitamin E zuständig ist, scheint immer noch offen zu sein. Zunächst wurde die Isoform CYP3A4 identifiziert (PARKER *et al.* 2000). Zwischenzeitliche Erkenntnisse lieferten gute Evidenz für CYP4F2 (SONTAG & PARKER 2002), während neueste Erkenntnisse wieder gegen CYP4F2 sprechen (persönliche Mitteilung von Francesco Galli (Abteilung für pharmazeutische Wissenschaften, Universität Perugia, Italien), 16th *Fat Soluble Vitamin Congress* in Paris 2017). In einem zweiten Schritt wird das α-Carboxychromanol (α-13'-COOH) über eine ω-Oxidation generiert. Die sukzessive Kürzung der Seitenkette über die β-Oxidation führt schlussendlich zum wasserlöslichen Endprodukt α-Carboxyhydroxyethylchromanol (α-3'-COOH oder α-CEHC).

Der Abbau des Vitamin E erfolgt in unterschiedlichen Kompartimenten (MUSTACICH *et al.* 2010), wobei der Transport der Metabolite innerhalb der Zelle, die Aufnahme in die jeweiligen Kompartimente und die beteiligten Enzyme noch ungeklärt sind. Zusätzlich erfolgt teilweise eine Modifikation der Metabolite durch Phase-II-Enzyme des Fremdstoffmetabolismus. So findet eine Sulfatierung der Metabolite parallel zum Metabolismus statt, z.B. entsteht 11'S-COOH (JIANG *et al.* 2007). FREISER & JIANG (2009b) geben zur rechnerischen Abschätzung der sulfatierten LCM einen

Faktor von 1,4 an, welcher mit der ermittelten Konzentration der LCM multipliziert wird. Ebenso wurden Glucuronide (SWANSON *et al.* 1999; ZHAO *et al.* 2010) und Glucoside (CHO *et al.* 2009) für die Metabolite beschrieben.

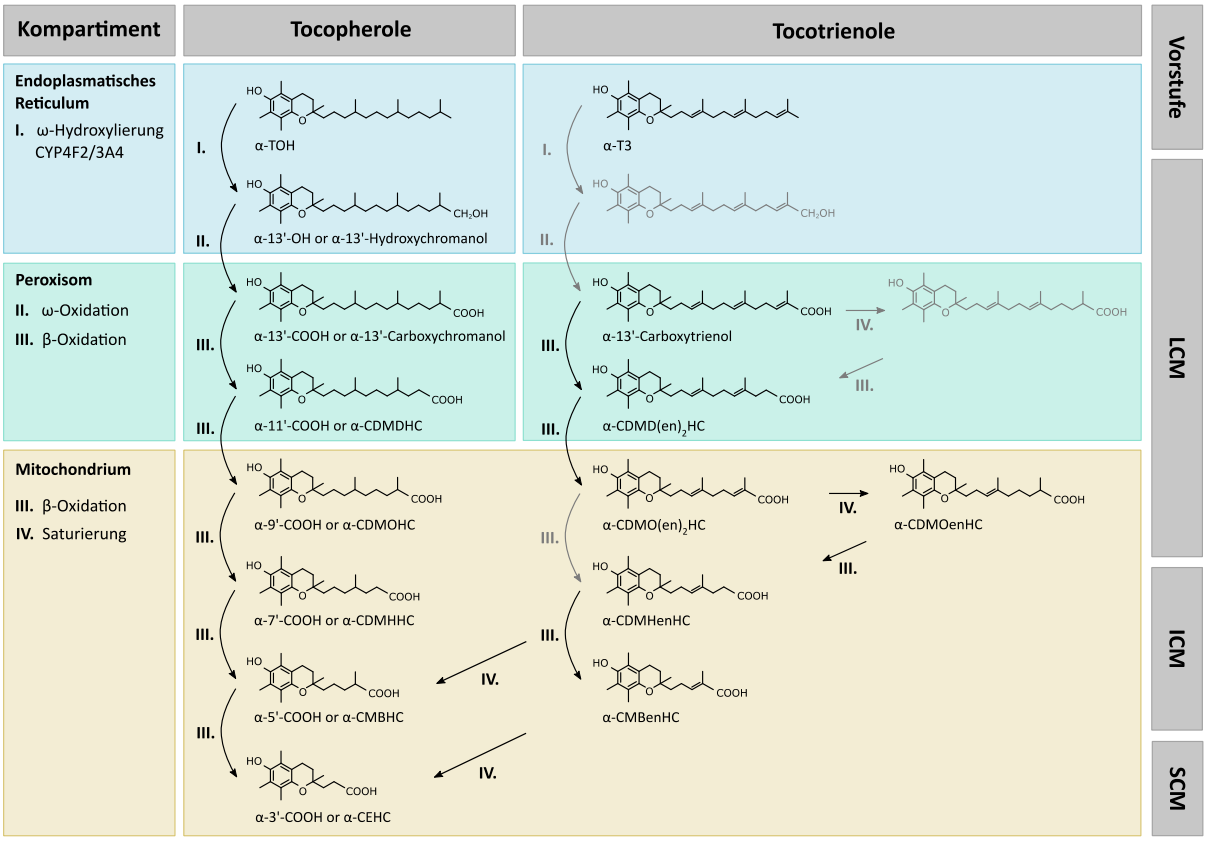


Abbildung 3: Hepatischer Metabolismus von Vitamin E (siehe Text für Erläuterungen).
Verwendete Abkürzungen: α-TOH, α-Tocopherol; α-T3, α-Tocotrienol; 13'-OH, 13'-Hydroxychromanol; 13'-COOH, 13'-Carboxychromanol; CYP4F2/3A4, Cytochrome-P450-abhängige Enzyme der Familie 4F2 oder 3A4; LCM, *long-chain metabolites*; ICM, *intermediate-chain metabolites*; SCM, *short-chain metabolites*; $CDMD(en)_2HC$, Carboxydimethyldecadienylhydroxychromanol; $CDMO(en)_2HC$: Carboxydimethyloctdienylhydroxychromanol; CDMHenHC, Carboxymethylhexenylhydroxychromanol; CMBenHC, Carboxymethylbutadienylhydroxychromanol; CDMDHC, Carboxymethyldecahydroxychromanol; CDMOHC, Carboxymethyloctylhydroxychromanol; CDMHHC, Carboxymethylhexylhydroxychromanol; CMBHC, Carboxymethylbutylhydroxychromanol; CEHC, Carboxyethylhydroxychromanol. Abbildung modifiziert nach Manuskript 6.

2 Langkettige Vitamin-E-Metabolite (LCM)

SONTAG & PARKER konnten 2002 erstmals unter anderem die langkettigen Metabolite des Vitamin E beschreiben und benannten diese der Nomenklatur folgend 13'-COOH. Diese Publikation legte den Grundstein für zahlreiche Arbeiten, die zum einen das Vorkommen der Metabolite in unterschiedlichen biologischen Materialen untersuchten und zum anderen die Analytik selbst optimierten. Abbildung 4 fasst die geleisteten Forschungsarbeiten zu den LCM knapp zusammen.

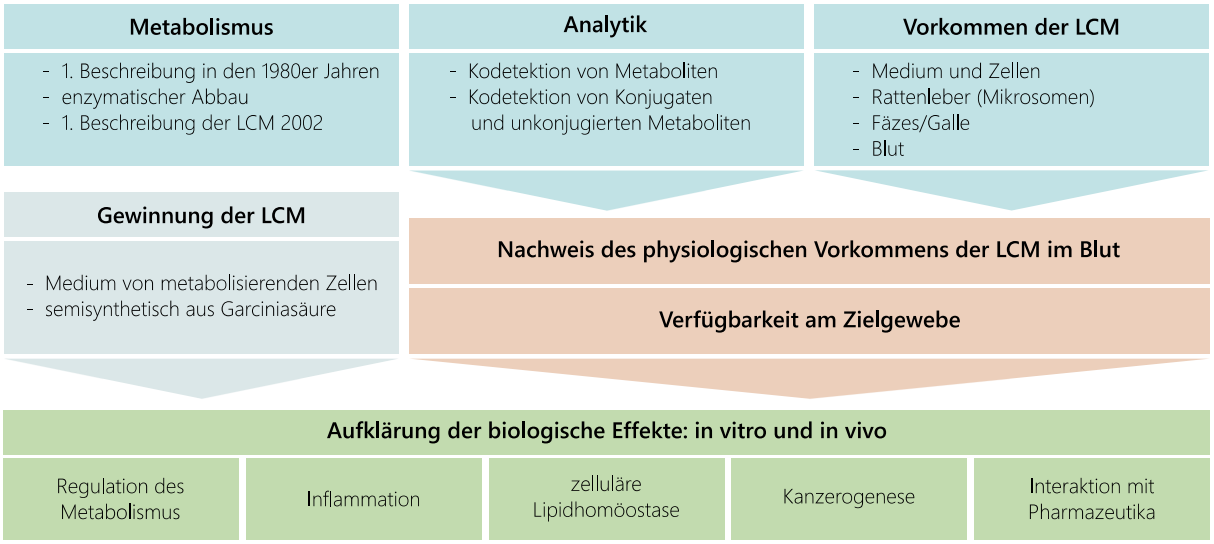


Abbildung 4: Forschungsarbeiten zu den LCM.
Verwendete Abkürzung: LCM, *long-chain metabolite*.

2.1 Analytik der LCM

Die Analytik der LCM ist in zahlreichen Publikationen beschrieben und weist im Detail ebenso viele Varianten auf (siehe Tabelle A1 im Anhang). Zwei Übersichtsarbeiten fassen diese Unterschiede anschaulich zusammen (BIRRINGER 2010; TORQUATO *et al.* 2016b). Die Probenmatrix variiert von Zellkulturproben (Zellen und Zellkulturmedium) über Blutproben (Serum oder Plasma) und Gewebeproben bis hin zu Fäzesproben. Das zu analysierende Probenvolumen ist von der Probenmatrix und von der Sensitivität der eingesetzten Methode abhängig. Für die Analyse von Blutproben ist das benötigte Probenvolumen insbesondere für große Humanstudien relevant, da hier das zur Verfügung stehende Probenmaterial häufig begrenzt ist. Je geringer das nötige Probenvolumen, umso einfacher lassen sich große Screening-Vorhaben ermöglichen.

Für die Messung der Metabolite ist zunächst eine Aufarbeitung der entsprechenden Proben nötig. Die Proben können enzymatisch oder chemisch dekonjugiert werden, um die Gesamtmenge der LCM (konjugiert und unkonjugiert) zu bestimmen. Zur enzymatischen Dekonjugation werden in der Regel Sulfatasen und Glucoronidasen eingesetzt, um die mengenmäßig relevantesten Konjugate zu lösen. Im Detail wurde die Verwendung der einzelnen Enzyme durch FREISER & JIANG (2009b) systematisch optimiert. Dabei spielen das explizite Enzym, die Inkubationsdauer, die Inkubationstemperatur und der eingesetzte pH-Wert eine entscheidende Rolle.

Anschließend werden die Metabolite aus der Probenmatrix extrahiert und im Bedarfsfall derivatisiert. Grundsätzlich werden zwei unterschiedliche Extraktionsmethoden eingesetzt: die Flüssig-Flüssig-Extraktion und die Flüssig-Festphasenextraktion. Das Lösungsmittelgemisch für die Flüssig-Flüssig-Extraktion richtet sich nach den zu analysierenden Metaboliten. Sollen nur die LCM analysiert werden, kann das Lösungsmittel in der Polarität so gewählt werden, dass es die Extraktion der LCM (z.B. durch Hexan) favorisiert. Sollen zeitgleich auch die TOH oder gar die kurzkettigen Metabolite (*short-chain metabolites*, SCM) extrahiert werden, müssen eventuell mehrere Extraktionsschritte mit Lösungsmittelgemischen unterschiedlicher Polaritäten miteinander kombiniert werden, um alle Metabolite bzw. Ausgangssubstanzen effizient zu

extrahieren (JIANG *et al.* 2015). Die Festphasenextraktion wurde bislang nur vereinzelt eingesetzt (YANG *et al.* 2010).

Die chromatographische Auftrennung erfolgt entweder mittels Gaschromatographie (GC) oder in der Flüssigchromatographie (*liquid chromatography*, LC oder *high pressure liquid chromatography*, HPLC). Die Detektion erfolgt je nach erwarteter LCM-Konzentration z.B. mittels Fluoreszenzdetektion (z.B. für Proben aus der Zellkultur) oder mittels Massenspektrometrie (MS). Technische Details werden in der Übersichtsarbeit von TORQUATO *et al.* (2016b) sehr anschaulich zusammengefasst.

Auch die Zahl der gleichzeitig detektierbaren Metabolite variiert stark und ist unter anderem von der Extraktionsmethode abhängig. Besonders problematisch ist der hohe Konzentrationsunterschied zwischen den TOH und den Metaboliten in z.B. Blutproben, die einen enorm breiten Detektionsbereich erforderlich machen. Meist wird zur Umgehung dieses Problems eine zweite chromatographische Auftrennung mit einer verdünnten Probe für die TOH benötigt (GIUSEPPONI *et al.* 2017).

2.2 Nachweis der LCM *in vitro* und *in vivo*

Der erste Nachweis der LCM erfolgte durch SONTAG & PARKER (2002) in HepG2-Zellen und in Mikrosomen, die aus Rattenleber gewonnen wurden. *In vitro* wurden die Metabolite außerdem in A549-Zellen nachgewiesen (FREISER & JIANG 2009a; JIANG *et al.* 2007; JIANG *et al.* 2008). *In vivo* werden die LCM auf Grund ihrer geringen Wasserlöslichkeit über die Galle und die Fäzes ausgeschieden und konnten dort auch detektiert werden (JIANG *et al.* 2015; ZHAO *et al.* 2010). Tabelle 2 gibt eine Übersicht über die in den jeweiligen Testmatrizes detektierten LCM. Nicht aufgeführt werden die Metabolite des β-TOH, da diese bislang nicht beschrieben wurden. Dies kann zum einen an der geringen physiologischen β-TOH-Konzentration liegen (0 - 0,2 µg/ml β-TOH im Serum; CHOW 1975), zum anderen ist die chromatographische Trennung von β- und γ-TOH schwierig, so dass beide Substanzen häufig koeluieren (SILUK *et al.* 2007). Zudem liegt γ-TOH in einer höheren Konzentration vor (0,7 - 2,7 µg/ml γ-TOH im Serum; CHOW 1975). Dies erschwert die Detektion von β-TOH und seiner möglichen Metabolite weiterhin.

Im Jahr 2014(a) konnten WALLERT *et al.* zum ersten Mal zeigen, dass α-13'-COOH physiologisch im humanen Blut zirkuliert. Im folgenden Jahr gelang CIFFOLILLI *et al.* (2015) der Nachweis, dass auch α-13'-OH nach siebentägiger Supplementation mit 1000 IU/d *RRR*-α-TOH im Serum vorliegt. Die Konzentration dieser Metabolite liegt im nanomolaren Bereich und konnte damals für α-13'-OH erst nach Supplementation zuverlässig detektiert werden (CIFFOLILLI *et al.* 2015). GIUSEPPONI *et al.* (2017) optimierten die LCM-Analytik und fanden neben den LCM auch bislang unidentifizierte Peaks, die möglicherweise ebenfalls zu den LCM gerechnet werden können. Die basale Konzentrationen für α-13'-OH geben GIUSEPPONI *et al.* (2017) mit ~0,8 nM und α-13'-COOH mit ~1,2 nM an (Konzentrationsangaben ohne unidentifizierte Peaks). Nach einer siebentägigen Supplementation mit 1000 IU/d *RRR*-α-TOH stiegen die Serumkonzentrationen auf ~8 nM für α-13'-OH und ~2,4 nM für α-13'-COOH.

Der Nachweis der LCM im humanen Blut zeigte zum ersten Mal, dass die LCM nicht ausschließlich über die Galle und Fäzes ausgeschieden werden, sondern physiologisch im Blut vorliegen. Damit

ist eine Zirkulation im gesamten Blutkreislauf realistisch und die Verfügbarkeit der Metabolite an Zielgeweben oder Zielzellen denkbar. Aus diesem Grund ist der *In-vitro*-Einsatz der LCM z.B. in extrahepatischen Zellen, wie Makrophagen, sinnvoll und für die Aufklärung der biologischen Funktion der Metabolite relevant.

Tabelle 2: Art und Ort der nachgewiesenen LCM in vivo und in vitro.

	α-								γ-								δ-							
	TOH				T3				TOH				T3				TOH				T3			
	M	B	F	L	M	B	F	L	M	B	F	L	M	B	F	L	M	B	F	L	M	B	F	L
9'-COOH																								
11'-COOH																								
13'-COOH																								
13'-OH																								
9'S-COOH																								
11'S-COOH																								
13'S-COOH																								

Farbig markierte Felder geben den Ort des detektierten Metabolits an. Die hier verwendete Definition der LCM entspricht der des Manuskriptes 6 (9'- bis 13'-LCM). **Verwendete Abkürzungen:** 9'-COOH, 9'-Carboxychromanol, (analog für 11'- und 13'-COOH); 9'S-COOH, 9'-Carboxychromanolsulfat, (analog für 11'S- und 13'S-COOH); 13'-COOH, 13'-Carboxychromanol; B, Blut; F, Fäzes; L, Leber; M, Medium; TOH, Tocopherol; T3, Tocotrienol.

2.3 Natürliche oder semisynthetische Gewinnung der LCM

Für den Einsatz der LCM in experimentellen Studien *in vivo* und *in vitro* müssen die Metabolite in ausreichenden Mengen und in hoher Reinheit vorliegen. In der Historie der LCM-Forschung wurden bislang dafür zwei Quellen genutzt. Zum einen wurden die Metabolite aus Zellkulturmedien isoliert; zum anderen uber einen Inhaltsstoff aus einer afrikanischen Bitternuss, der *Garcinia kola*, hergestellt.

Die bei der Isolation der LCM aus Zellkulturmedien verwendeten Zellen (z.B. HepG2 oder A549) können nachweislich Vitamin E verstoffwechseln und wurden mit den entsprechenden TOH inkubiert (JIANG *et al.* 2008; JIANG *et al.* 2011). Damit machte man sich die Fähigkeit der Leberzellen zum TOH-Metabolismus zu Nutze. Der große Vorteil dieser Methode liegt in der enantiomerreinen Synthese der Metabolite, während die Bildung von Metaboliten unterschiedlicher Länge (z.B. 13'-COOH und 9'-COOH) nachteilig für die Aufklärung der spezifischen Effekte der einzelnen Metabolite ist.

Für eine semisynthetische Gewinnung der LCM aus *Garcinia kola* legten MAZZINI *et al.* (2009) den Grundstein. Die Nuss enthält Garciniasäure (*garcinoic acid,* GA; δ-T3-13'-COOH, LCM des δ-T3), welche zunächst isoliert und anschließend mittels Syntheseschritten zu δ-13'-COOH, δ-13'-OH und α-13'-COOH sowie α-13'-OH umgewandelt werden kann (siehe Abbildung 5). Vorteil dieser LCM-Gewinnung ist die relativ gute Verfügbarkeit der Nüsse und die spezifische Synthese einzelner Metabolite, während das gebildete Enantiomergemisch, welches bei der Hydrierung der ungesättigten Seitenkette entsteht, nachteilig für die experimentellen Studien ist. Möglicherweise

ist dadurch die für spezifische biologische Effekte benötigte Konzentration der LCM höher als bei einer enantiomerreinen Substanz. In einer Arbeit aus dem Jahr 2016 untersuchten JANG *et al.* u.a. den Einfluss der Gewinnung der Metabolite auf ihre biologische Aktivität und kamen zu dem Schluss, dass für die untersuchten Enzyme die jeweiligen IC_{50}-Werte (mittlere inhibitorische Konzentration, IC_{50}) vergleichbar sind, unabhängig davon, ob die Metabolite aus Zellkulturmedien isoliert oder semisynthetisch aus der *Garcinia kola* gewonnen wurden. Daher ist der bevorzugte Einsatz der semisynthetisch erzeugten LCM für die Experimente gerechtfertigt (BIRRINGER *et al.* 2010; CIFFOLILLI *et al.* 2015; JANG *et al.* 2016; PODSZUN *et al.* 2017; TORQUATO *et al.* 2016a; TORQUATO *et al.* 2016b; WALLERT *et al.* 2014a; WALLERT *et al.* 2015).

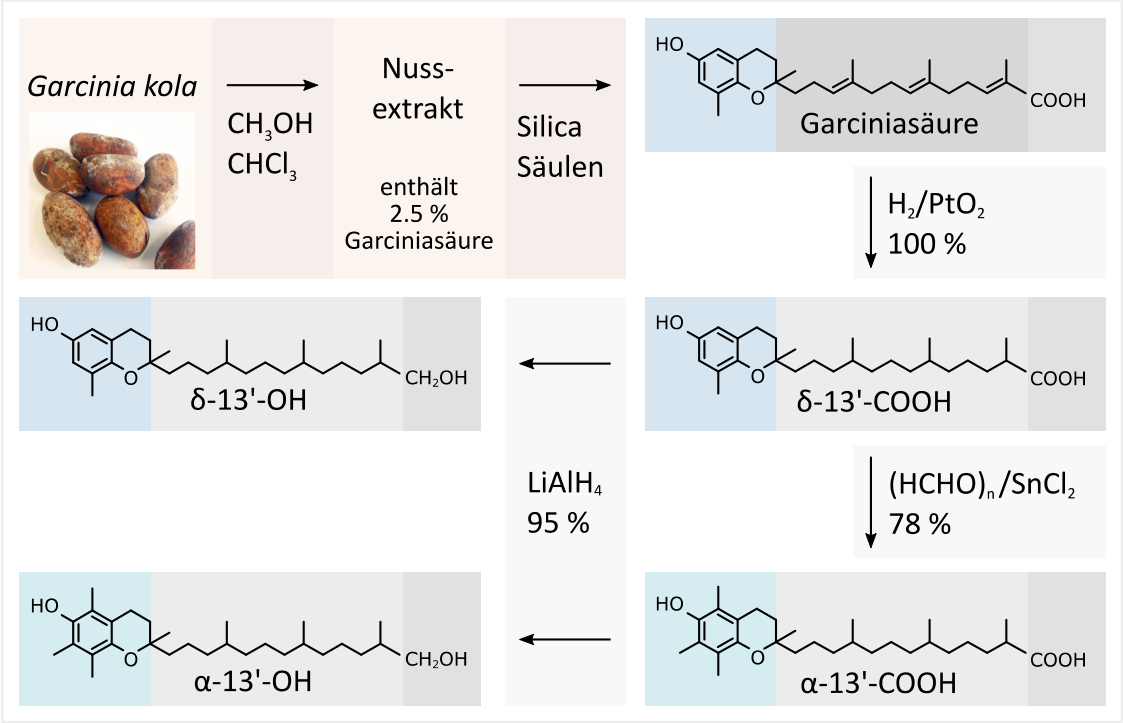


Abbildung 5: Semisynthetische Gewinnung der LCM (siehe Text für Erläuterungen).
Verwendete Abkürzungen: α-13'-COOH, α-13'-Carboxychromanol, (analog für δ-13'-COOH); α-13'-OH, α-13'-Hydroxychromanol (analog für δ-13'-OH). Abbildung modifiziert nach Manuskript 1.

2.4 Biologische Effekte der LCM

Erstmals 2008 wurden biologische Wirkungen der LCM beschrieben (JIANG *et al.* 2008). Seither wächst die Zahl der Publikationen auf diesem Feld stetig und es zeigte sich, dass die LCM pleiotrope Effekte vermitteln. Bislang konzentrierten sich die Forschergruppen auf den Einfluss der LCM auf die Inflammation, die Kanzerogenese, die zelluläre Lipidhomöostase, den Metabolismus und auf die Interaktion mit Pharmazeutika. Tabelle 3 gibt einen Überblick über die bekannten biologischen Effekte der LCM.

Tabelle 3: Biologische Effekte der LCM und der GA.

Autoren	Beobachteter Effekt	Substanz
Antiinflammatorische Effekte		
JIANG *et al.* 2008	Inhibition der Aktivität von COX2	γ-13′-COOH δ-13′-COOH δ-9′-COOH
CIFFOLILLI *et al.* 2015	Hemmung der Transkription und Funktion von COX2	α-13′-OH
JANG *et al.* 2016	Inhibition der Aktivität von COX2	δ-13′-COOH GA
CIFFOLILLI *et al.* 2015	Hemmung der Transkription und Funktion von iNOS	α-13′-OH
JIANG *et al.* 2011	Inhibition der Aktivität von 5-LO	δ-13′-COOH
JANG *et al.* 2016	Inhibition der Aktivität von 5-LO	δ-13′-COOH GA
Antikanzerogene Effekte		
BIRRINGER *et al.* 2010	Induktion der Apoptose Induktion der mitochondrialen ROS-Bildung Reduktion des mitochondrialen Membranpotentials	α-13′-OH α-13′-COOH δ-13′-OH δ-13′-COOH
MAZZINI *et al.* 2009	Antiproliferative Effekte	α-13′-OH δ-13′-COOH GA
JANG *et al.* 2016	Antiproliferative Effekte Induktion der Apoptose Induktion der Autophagie Regulation der Phospholipide Tumore: Größe und Anzahl reduziert	δ-13′-COOH GA
Zelluläre Lipidhomöostase		
WALLERT *et al.* 2014a	Induktion der Expression von CD36	α-13′-OH α-13′-COOH
Interaktion mit Pharmazeutika		
PODSZUN *et al.* 2017	Induktion der P-gp-Expression und Aktivität Aktivierung von PXR	α-13′-COOH
Regulation des Metabolismus		
TORQUATO *et al.* 2016a	Induktion der CYP4F2-Expression	α-13′-OH

Verwendete Abkürzungen: 5-LO, 5-Lipoxygenase; α-13′-COOH, α-13′-Carboxychromanol, (analog für δ-13′-COOH); α-13′-OH, α-13′-Hydroxychromanol, (analog für δ-13′-OH); CD36, *cluster of differentiation 36*; COX2, Cyclooxygenase 2; CYP4F2, Cytochrom-P450-abhängiges Enzym der Familie 4F2; GA, Garciniasäure; iNOS, *inducible nitric oxide synthase*; LCM, *long-chain metabolite*; NO, *nitric oxide*; P-pg, P-Glykoprotein; ROS, *reactive oxygen species*; PXR, *pregnane X receptor*.

2.4.1 Antiinflammatorische Effekte

Die Inflammation ist ein komplexes Geschehen, das pharmazeutisch über zahlreiche Angriffspunkte abgeschwächt bzw. aufgelöst werden kann. So stehen z.B. Hemmstoffe für die Cyclooxygenasen (COX, z.B. Aspirin) oder die 5-Lipoxygenase (5-LO, z.B. Zileuton) zur Verfügung (MEIRER *et al.* 2014). Diese Enzyme sind an der Umsetzung von Arachidonsäure zu proinflammatorischen Mediatoren, wie z.B. Prostaglandinen (PG) oder Leukotrienen (LT), beteiligt (PEREIRA-LEITE *et al.* 2017). Weiterhin wird die Expression der induzierbaren Stickstoffmonoxidsynthase (*inducible nitric oxide synthase*, iNOS) durch immunologische oder mikrobielle Stimuli aktiviert, welche dann lokal hohe Konzentrationen an Stickstoffmonoxid produziert und dadurch (in Abhängigkeit von den Gegebenheiten) positive und negative Effekte auf den Krankheitsverlauf haben kann (LIND *et al.* 2017). Die experimentellen Arbeiten zur Aufklärung der antiinflammatorischen Effekte der LCM konzentrieren sich daher vorrangig auf die Regulation der Expression und der Funktion der genannten Enzyme.

JIANG *et al.* (2008) inkubierten A549-Zellen, welche zum Vitamin-E-Metabolismus fähig sind, mit unterschiedlichen Vitamin-E-Formen und zeigten eine Inhibition der arachidonsäureinduzierten Aktivität der Cyclooxygenasen. Die Effekte von Vitamin E waren weniger deutlich, wenn der Vitamin-E-Metabolismus z.B. durch Sesamin inhibiert wurde. Daher schlossen JIANG *et al.* (2008), dass die Regulation der COX zumindest zum Teil durch Metabolite des Vitamin E vermittelt werden. Zur Bestätigung wurden Zellen mit γ-TOH und δ-TOH inkubiert und das entstehende LCM-angereicherte Inkubationsmedium für eine weitere Inkubation genutzt. Dabei zeigte das δ-TOH-behandelte Medium eine potentere Hemmung der arachidonsäureinduzierten COX2-Aktivität als das γ-TOH-behandelte. Da δ-13'-COOH und δ-9'-COOH den mengenmäßig größten Anteil an den in A549-Zellen gebildeten LCM ausmachen, wurden diese aus dem Zellkulturmedium isoliert und am isolierten Enzym getestet. Dabei war die Hemmeffizienz von COX2 bei einer mittleren inhibitorischen Konzentration (*half maximal inhibitory concentration*, IC_{50}) von 4 - 6 µM für beide Metabolite im zellbasierten Assay sehr ähnlich, während am isolierten Enzym δ-13'-COOH COX1 und COX2 effektiver inhibierte als δ-9'-COOH. Der Einfluss der Sulfatierung wurde ebenfalls untersucht und es konnte nachgewiesen werden, dass die unkonjugierten Metabolite die Cyclooxygenasen effektiv hemmen, während ihre Konjugate diesbezüglich keine Wirkung zeigten. Für diese Versuche nutzten die Autoren γ-T3 inkubierte Zellen, welche nach 24 h vorrangig unkonjugierte LCM produzieren. Nach 72 h Inkubationszeit hingegen liegt der Großteil der LCM sulfatiert vor, während die Gesamtmenge der LCM vergleichbar ist. Aus den jeweiligen Medien wurden die Metabolite isoliert und *in vitro* eingesetzt. In Computersimulationen konnte eine stärkere Bindung von 13'-COOH als von 9'-COOH an COX1 gezeigt werden (JIANG *et al.* 2008).

JIANG *et al.* zeigten im Jahr 2011 die inhibitorische Wirkung von δ-13'-COOH auf die 5-LO. In HL-60-Zellen hemmte der Metabolit die A23187-induzierte Leukotrien B_4 (LTB_4)-Bildung effektiv mit einem IC_{50}-Wert von 4 - 7 µM. Am isolierten Enzym übertraf die Hemmung von 5-LO durch δ-13'-COOH (IC_{50}: 0,5 - 1 µM) die des synthetischen Inhibitors Zileuton (IC_{50}: 3 - 5 µM).

JANG *et al.* bestätigten im Jahr 2016 die Ergebnisse aus den vorangegangenen Studien (JIANG *et al.* 2008; JIANG *et al.* 2011) und zeigten, dass δ-13'-COOH unabhängig von seiner Herkunft (isoliert aus Zellkulturmedium oder semisynthetisch gewonnen aus *Garcinia kola*) eine vergleichbare

inhibitorische Wirkung auf die untersuchten Enzyme (COX2 und 5-LO) aufweist (JANG *et al.* 2016). Im Vergleich zu GA zeigte δ-13′-COOH eine effektivere Hemmung der COX2-Aktivität, aber eine geringere Hemmung der 5-LO-Aktivität.

CIFFOLILLI *et al.* zeigten 2015 für α-13′-OH eine antiinflammatorische Aktivität. So wurde die transkriptionelle Kontrolle inflammatorischer Zytokine (*tumor necrosis factor α*, Tnfα; Interleukin 1β, Il1β; Il10), sowie die transkriptionelle und funktionelle Hemmung von Cox2 und iNos in Lipopolysaccharid (LPS)-stimulierten murinen RAW264.7-Makrophagen gezeigt.

2.4.2 Antikanzerogene Effekte

Antiinflammatorisch wirksamen Naturstoffen wird das Potential zur Hemmung der Krebsinitiation zugeschrieben (ARAVINDARAM & YANG 2010). Unter anderem können Prozesse, wie die Modulation der Zellproliferation oder die Apoptose beeinflusst werden, aber auch die Resistenz von Tumorzellen gegenüber einer antitumoralen Therapie kann reduziert werden (PARK & SURH 2017).

Wie schon für die GA beschrieben wurde, zeigten MAZZINI *et al.* (2009) auch für δ-13′-COOH und α-13′-OH eine antiproliferative Wirkung. JANG *et al.* (2016) zeigten, dass die Zellvitalität von Kolonkrebszellen (HCT-116 und HT-29) durch δ-13′-COOH und GA reduziert wird und schlossen deshalb, dass die Proliferation von Krebszellen durch die untersuchten Metabolite gehemmt werden könnte.

BIRRINGER *et al.* (2010) nutzten die semisynthetisch gewonnenen Metabolite (α-13′-OH, α-13′-COOH, δ-13′-OH, δ-13′-COOH) zur Aufklärung ihrer biologischen Effekte in HepG2-Zellen. Dabei nahm die Zahl apoptotischer Zellen unter LCM-Behandlung zu. Die 13′-Carboxy-chromanole induzierten Caspase-3, -7 und -9 prominent, während eine schwächere Induktion unter δ-13′-OH-Behandlung bzw. keine Induktion unter α-13′-OH-Behandlung sichtbar war. Ein sehr ähnliches Bild zeigte sich für die Induktion der Poly-ADP-Ribose-Polymerase-1 (PARP-1)-Spaltung. Da Apoptose mit der Bildung von reaktiven Sauerstoffspezies (*reactive oxygen species*, ROS) assoziiert sein kann, wurde sowohl die intrazelluläre, als auch die intramitochondriale ROS-Bildung untersucht, welche jeweils durch 13′-COOH induziert wurde. Das mitochondriale Membranpotential, das als Marker für mitochondrial bedingte Apoptose gewertet wird.

Ein ähnliches Bild zeigte sich für δ-13′-COOH und GA in Kolonkrebszellen (JANG *et al.* 2016). Auch hier induzierten die untersuchten Metabolite die frühe und späte Apoptose, die Spaltung von PARP und die Expression von LC-3-II, einem Marker für Autophagie. Die Effektgröße war hierbei für δ-13′-COOH größer als für die GA. Es wurde in COX2-negativen Zellen außerdem getestet, ob die antikanzerogene Wirkung der Metabolite durch COX2 und 5-LO vermittelt wird, da die aus Arachidonsäure gebildeten Eicosanoide bekannt sind für eine Begünstigung der Kolonkrebs-entwicklung. Die Induktion der Apoptose war in den COX2-negativen Zellen etwas schwächer ausgeprägt, daher schlussfolgerten die Autoren, dass die Effekte z.T. von COX2 unabhängig vermittelt werden.

Der Phospholipidgehalt in der Zelle wird durch δ-13′-COOH und GA reguliert (JANG *et al.* 2016). Zu einem frühen Zeitpunkt (2 h) erhöhte die Behandlung mit δ-13′-COOH die Konzentration an Dihydroceramiden und verminderte die Konzentration der Ceramide, was sich in einer verminderten Aktivität (~60 % Aktivität in HCT-116 Zellen) des entsprechenden Enzyms

widerspiegelte (Dihydroceramiddesaturase). Nach einer Inkubationszeit von 8 - 16 h wurden höhere Konzentrationen an Dihydroceramiden, Dihydrosphingosinen und Ceramiden im Vergleich zu Kontrollzellen gemessen, aber geringere Konzentrationen an Sphingomyelin. Zu diesem Beobachtungszeitpunkt wurden außerdem Autophagie und Apoptose beobachtet. Durch eine Inhibition der Sphingosinbiosynthese (Myriocin) wurde der Effekt von δ-13'-COOH und GA auf die LC3-II-Expression abgeschwächt, was darauf hindeutet, dass die erhöhte Konzentration der Dihydroceramide und Dihydrosphingosine an der 13'-COOH-induzierten Autophagie eine Rolle spielen könnte. Ob die erhöhte Konzentration der Ceramide durch eine vermehrte Hydrolyse von Sphingomyelin durch Sphingomyelinasen (SMasen) erklärt werden könnte, wurde mittels spezifischen SMase-Inhibitoren getestet und bestätigt. Eine erhöhte Sphingomyelinkonzentration könnte somit durch eine erhöhte SMase-Aktivität zu den antiproliferativen Effekten der 13'-COOH beitragen.

2.4.3 Zelluläre Lipidhomöostase

Die zelluläre Lipidhomöostase ist ein Zusammenspiel aus vielfältigen Prozessen, wie der zellulären Aufnahme, Verteilung, Speicherung und des Exportes von Fettsäuren oder Sterolderivaten und spielt bei der Entstehung von atherosklerotischen Plaques eine wichtige Rolle (siehe Manuskript 5). An der Aufnahme beteiligte Proteine sind z.B. der Rezeptor CD36 (*cluster of differentiation 36;* COLLOT-TEIXEIRA *et al.* 2007) oder die Fettsäuretransportproteine (*fatty acid transport proteins*, FATPs; BLACK *et al.* 2016). Die intrazelluläre Speicherung der Fettsäuren erfolgt nach ihrer Veresterung zu Neutral- oder Phospholipiden in Lipidtropfen, die von lipidtropfenassoziierten Proteinen wie Adipophilin (*adipose differentiation related protein*, ADRP oder PLIN2) umgeben sind (WALTHER & FARESE 2012). Der Export von z.B. Cholesterol wird von Transportern, wie ABCA1 (*Adenosine triphosphate (ATP)-binding cassette transporter A1*) bewerkstelligt (NAGAO *et al.* 2011).

WALLERT *et al.* untersuchten im Jahr 2014(a) den Einfluss der α-LCM auf die Schaumzellbildung und nutzten dafür humane THP-1-Monozyten bzw. daraus differenzierte Makrophagen. Unter basalen Bindungen induzierten α-13'-OH und α-13'-COOH die mRNA- (*messenger ribonucleic acid*) und Proteinexpression von CD36. Um die THP-1-Makrophagen als Modell für die Schaumzellbildung zu nutzen, wurden die Zellen mit oxidiertem LDL (oxLDL) inkubiert. Dies bewirkte eine Induktion der CD36-Proteinexpression. Wurde zeitgleich auch mit den α-LCM inkubiert, erhöhten die Metabolite die schon durch oxLDL induzierte CD36-Proteinexpression weiter. Die zelluläre Aufnahme von oxLDL, die phagozytotische Aktivität und die Akkumulation der Neutrallipide wurden hingegen reduziert.

2.4.4 Interaktionen mit Pharmazeutika

Zur Regulation der zellulären Aufnahme von Molekülen verfügt der Organismus u.a. über Exporter, die z.B. Medikamente wieder aus den Zellen herausschleusen können (z.B. P-Glykoprotein, P-gp) (DEWANJEE *et al.* 2017). In Tumorzellen ist eine Hemmung dieser Transporter erwünscht, um die antitumoralen Medikamente zielgerichtet zu applizieren. Im Falle einer Aktivierung dieser Transporter muss unter Umständen mit einer verminderten Aufnahme von Medikamenten gerechnet werden.

PODSZUN *et al.* (2017) untersuchten die Wirkung von Vitamin-E-Formen und -Metaboliten (α-TOH, α-T3, α-13′-COOH, α-CEHC, γ-TOH, γ-T3, γ-CEHC und Plastochromanol-8) auf die P-gp-Expression in der humanen kolorektalen Adenomkrebszelllinie LS 180. Lediglich α-13′-COOH und γ-T3 konnten die Expression und Aktivität von P-gp induzieren. Um einen Einblick in die dahinterliegende Regulation zu erhalten, führten sie einen PXR-Reportergen-Assay (*pregnane X receptor*, PXR) durch und fanden eine Aktivierung von PXR durch α-T3, α-13′-COOH und γ-T3.

2.4.5 Regulation des Metabolismus

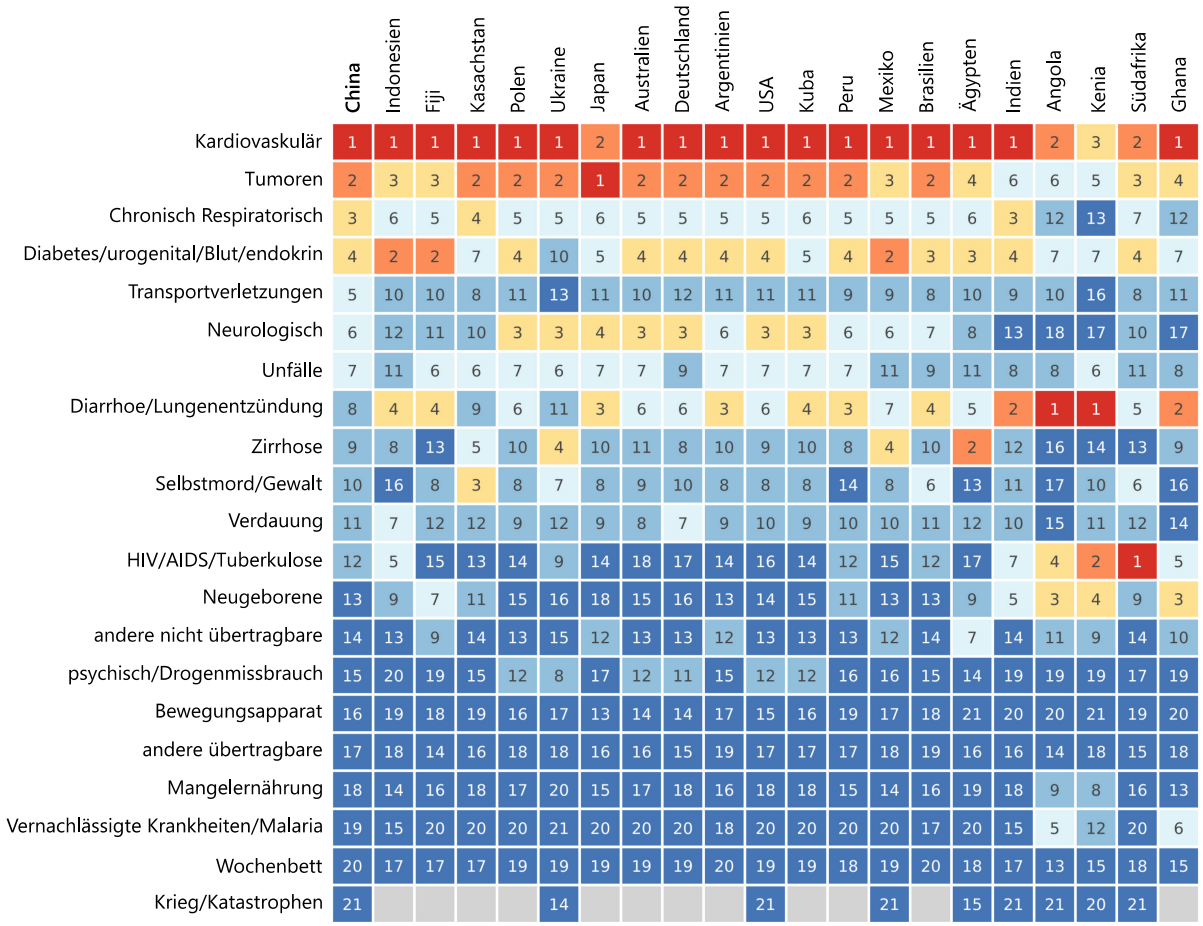
In einem Tagungsbeitrag präsentierten TORQUATO *et al.* (2016a) die Effekte von α-13′-OH auf die Regulation von CYP4F2 und den nukleären Rezeptor PPARγ (*peroxisome proliferator-activted receptor γ*) in HepG2-Zellen. Dabei zeigte der Metabolit eine Induktion auf Proteinebene, die mindestens 1,5-fach größer war als die des α-TOH. Möglicherweise deutet sich hier ein positiver Rückkopplungsmechanismus des Vitamin-E-Metabolismus an.

3 Atherogenese

Ein Teil der untersuchten biologischen Prozesse, die durch die LCM beeinflusst werden, können thematisch unterschiedlichen Krankheiten zugeordnet werden. So liefert bspw. die Regulation der Inflammation und der zellulären Lipidhomöostase Einblicke in Prozesse, die in der Atherogenese eine Rolle spielen. Wie bereits im Vorwort erwähnt, wurde der Einfluss von Vitamin E auf kardiovaskuläre Prozesse bzw. Endpunkte vielfach *in vitro* und in Humaninterventionsstudien untersucht. Die teils widersprüchlichen Ergebnisse könnten durch den hepatischen Metabolismus von Vitamin E im Menschen erklärt werden, welcher *in vitro* (in Abhängigkeit von der verwendeten Zelllinie) nicht zu erwarten ist. Diese Thematik wird im Manuskript 5 aufgegriffen. Die Übersichtsarbeit gibt einen Überblick über die Entstehung der Atherosklerose, fasst die *In-vivo*- und *In-vitro*-Daten zum Einfluss des Vitamin E zusammen und liefert einen Erklärungsansatz für die Diskrepanzen durch eine mögliche Beteiligung der LCM. Daher wird zur tiefgehenden Lektüre auf dieses Review verwiesen und an dieser Stelle lediglich kurz die medizinische Relevanz und wirtschaftliche Bedeutung der kardiovaskulären Erkrankungen dargelegt.

Kardiovaskuläre Erkrankungen sind weltweit mit 17,9 Mio. Todesfällen (2015) die Haupttodesursache (WANG *et al.* 2016). Abbildung 6 gibt einen Überblick über die nach Krankheitsart sortierten Todesfälle. In Deutschland erlagen im Jahr 2015 etwa 39 % der Verstorbenen Herz-Kreislauf-Erkrankungen (Statistisches Bundesamt 2017). Damit liegt Deutschland im weltweiten Vergleich nach Angaben des *Institute for Health Metrics and Evaluation* (IHME) etwa im Mittelfeld (IHME 2016). Für die Krankenkassen entstehen in Deutschland jährlich Kosten von etwa 37 Mrd. € bei der Behandlung von Herz-Kreislauf-Leiden (15 % der Gesamtkosten, Krankheitsart mit den höchsten Behandlungskosten, Statistisches Bundesamt 2017).

Aufgrund der hohen Anzahl an Todesfällen und der hohen Kosten für das Gesundheitswesen ist die Motivation hoch, eine effektive und akzeptierbare Prävention und Behandlung von kardiovaskulären Erkrankungen zur Verfügung zu stellen. Für dieses Vorhaben ist jedoch das Verständnis der zugrundeliegenden Mechanismen essentiell.



	China	Indonesien	Fiji	Kasachstan	Polen	Ukraine	Japan	Australien	Deutschland	Argentinien	USA	Kuba	Peru	Mexiko	Brasilien	Ägypten	Indien	Angola	Kenia	Südafrika	Ghana
Kardiovaskulär	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	3	2	1
Tumoren	2	3	3	2	2	2	1	2	2	2	2	2	2	3	2	4	6	6	5	3	4
Chronisch Respiratorisch	3	6	5	4	5	5	6	5	5	5	5	6	5	5	5	6	3	12	13	7	12
Diabetes/urogenital/Blut/endokrin	4	2	2	7	4	10	5	4	4	4	4	5	4	2	3	3	4	7	7	4	7
Transportverletzungen	5	10	10	8	11	13	11	10	12	11	11	11	9	9	8	10	9	10	16	8	11
Neurologisch	6	12	11	10	3	3	4	3	3	6	3	3	6	6	7	8	13	18	17	10	17
Unfälle	7	11	6	6	7	6	7	7	9	7	7	7	7	11	9	11	8	8	6	11	8
Diarrhoe/Lungenentzündung	8	4	4	9	6	11	3	6	6	3	6	4	3	7	4	5	2	1	1	5	2
Zirrhose	9	8	13	5	10	4	10	11	8	10	9	10	8	4	10	2	12	16	14	13	9
Selbstmord/Gewalt	10	16	8	3	8	7	8	9	10	8	8	8	14	8	6	13	11	17	10	6	16
Verdauung	11	7	12	12	9	12	9	8	7	9	10	9	10	10	11	12	10	15	11	12	14
HIV/AIDS/Tuberkulose	12	5	15	13	14	9	14	18	17	14	16	14	12	15	12	17	7	4	2	1	5
Neugeborene	13	9	7	11	15	16	18	15	16	13	14	15	11	13	13	9	5	3	4	9	3
andere nicht übertragbare	14	13	9	14	13	15	12	13	13	12	13	13	13	12	14	7	14	11	9	14	10
psychisch/Drogenmissbrauch	15	20	19	15	12	8	17	12	11	15	12	12	16	16	15	14	19	19	19	17	19
Bewegungsapparat	16	19	18	19	16	17	13	14	14	17	15	16	19	17	18	21	20	20	21	19	20
andere übertragbare	17	18	14	16	18	18	16	16	15	19	17	17	17	18	19	16	16	14	18	15	18
Mangelernährung	18	14	16	18	17	20	15	17	18	16	18	18	15	14	16	19	18	9	8	16	13
Vernachlässigte Krankheiten/Malaria	19	15	20	20	20	21	20	20	20	18	20	20	20	20	17	20	15	5	12	20	6
Wochenbett	20	17	17	17	19	19	19	19	19	20	19	19	18	19	20	18	17	13	15	18	15
Krieg/Katastrophen	21					14					21			21		15	21	21	20	21	

Abbildung 6: Haupttodesursachen im Jahr 2015.

Angegeben ist der Rang der Todesursache basierend auf der Anzahl der Todesfälle (pro 100 000 Verstorbene) je Krankheitsart sortiert nach Ländern. Abbildung modifiziert nach *Global Burden of Disease Compare Data Visualization* (IHME 2016).

4 Ziel der Arbeit

Das Bestreben der Forscher rund um das Thema der LCM ist die Aufklärung ihrer biologischen Bedeutung und physiologischen Eigenschaften. Auf zahlreichen Themengebieten (Kanzerogenese, Interaktion mit Pharmazeutika, Inflammation, u.a.; siehe Kapitel 2.4) wurden bereits Effekte beschrieben, die jeweils einen Beitrag zum Gesamtwirkbild der LCM liefern.

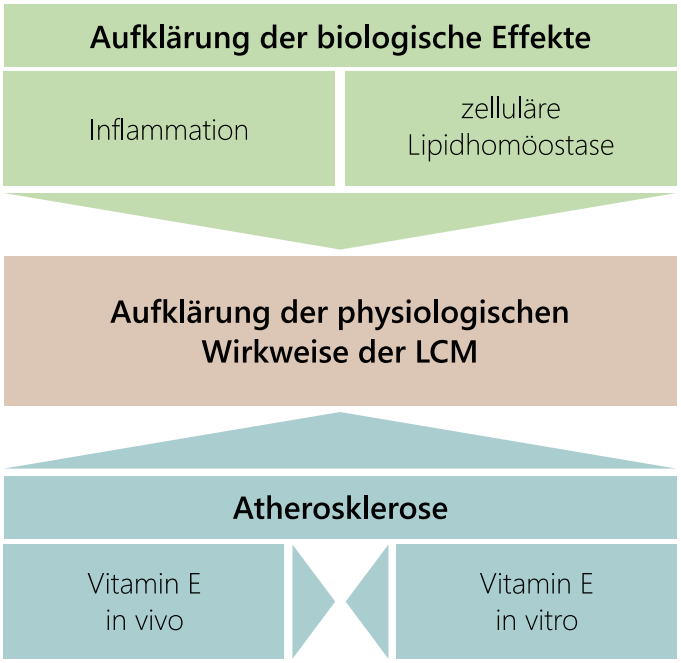


Abbildung 7: Physiologische Wirkweise der LCM im Kontext der Atherosklerose.
Verwendete Abkürzung: LCM, *long-chain metabolites*.

Die vorliegende Arbeit reiht sich in die vorangegangenen Arbeiten ein und untersucht die Wirkung der LCM im Kontext der Atherosklerose. Im Detail wurde ihr Einfluss auf die Inflammation und auf die zelluläre Lipidhomöostase untersucht. Darüber hinaus legt diese Arbeit einen Grundstein zur Aufklärung der physiologischen Wirkweise der LCM. Durch einen systematischen Vergleich von Leitsubstanzen soll hierbei der Einfluss der LCM-Substrukturen auf ihre Funktion geprüft werden und somit ein erster Schritt zur Identifikation eines putativen Rezeptors bzw. zugrundeliegende Signalwege ermöglicht werden.

5 Übersicht zu den Manuskripten

In der vorliegenden Arbeit werden Publikationen und Manuskripte zu den LCM und eng verknüpften Themen dargestellt. Die Reihung der Manuskripte basiert auf mehreren Gliederungsebenen. So werden zunächst die Originalarbeiten und dann die Übersichtsarbeiten vorgestellt, welche in sich wiederum nach dem Eigenanteil der Autorin bzw. thematisch sortiert sind. Nachfolgendes Fließschema ordnet die Gesamtheit der Manuskripte hingegen in eine logische Reihenfolge und gibt einen Überblick über ihre thematische Verknüpfung.

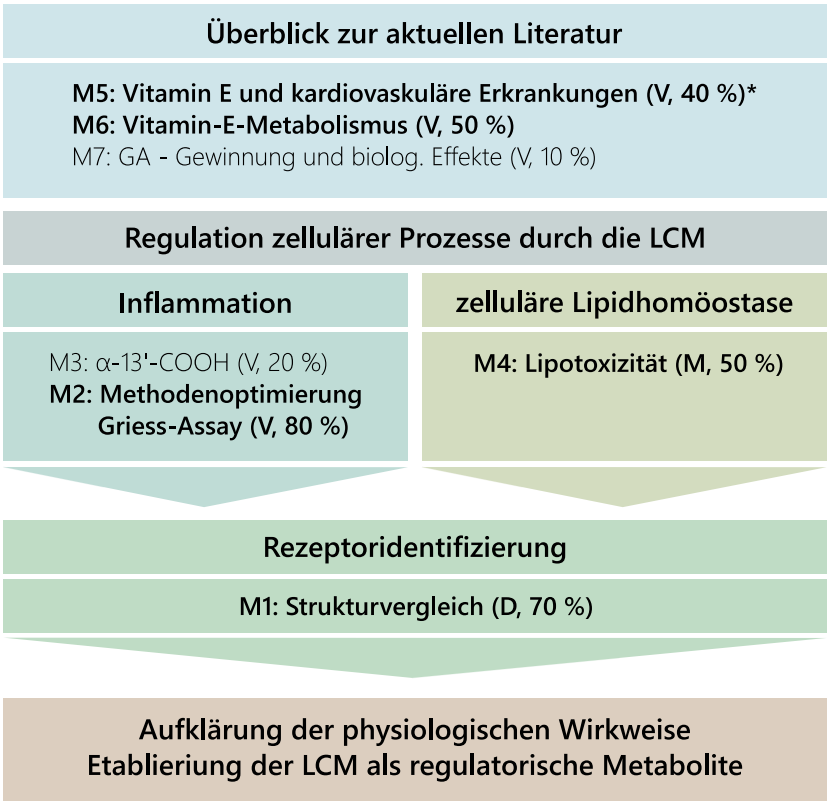


Abbildung 8: Übersicht über die Manuskripte dieser Arbeit.
Verwendete Abkürzungen: α-13'-COOH, α-13'-Carboxychromanol; (D), im Druck; (E), eingereicht; GA, Garciniasäure; LCM, *long-chain metabolite*; M, Manuskript; (M), in Manuskriptform; (V), veröffentlicht; fett markierte Manuskripte sind Erstautorenschaften (*, geteilt); prozentuale Angaben sind die Eigenanteile.

5.1 Manuskript 1 (im Druck)

Structure-function relationship studies *in vitro* reveal distinct and specific effects of long-chain metabolites of vitamin E

Schmölz L, Wallert M, Rozzino N, Cignarella A, Galli F,
Glei M, Werz O, Koeberle A, Birringer M, Lorkowski S

Molecular Nutrition and Food Research (2017); im Druck
Akzeptiert am 22.08.2017

Beitrag zum Manuskript

- Erstellung des Konzeptes
- Durchführung von quantitativer Reverse-Transkriptase-Polymerasekettenreaktion (*quantitative reverse transcriptase polymerase chain reaction*, RTq-PCR), der Western-Blots, der Versuche am Durchflusszytometer und der Griess-Assays
- Optimierung des Griess-Assays (vgl. Manuskript 2)
- Einarbeitung und Betreuung von Nicolò Rozzino als Masterstudent (Erasmus)
- Datenanalyse
- Evaluierung der Daten
- Erstellung des Manuskriptes

Eigenanteil am Manuskript: 70 %

Hauptaussagen des Manuskriptes 1

Diese Originalarbeit untersucht systematisch den Einfluss von LCM-Substrukturen auf deren biologische Aktivität. So werden die Effekte der Stellvertreter für Substrukturen (Seitenkette: Pristansäure, Ringsystem: α-CEHC) und der LCM-Vorstufen (α-TOH und δ-TOH) mit den Effekten der LCM (α-13'-OH, α-13'-COOH, δ-13'-OH, δ-13'-COOH) verglichen. Dabei zeigt sich, dass die LCM in niedrigeren Konzentrationen deutlichere Effekte in der Regulation von iNos oder CD36 vermitteln als die anderen Substanzen. Somit zeigen sich distinkte Effekte für die LCM, welche die Beteiligung spezifischer Signalwege nahelegen.

5.2 Manuskript 2 (veröffentlicht)

Optimized incubation regime for nitric oxide measurements in murine macrophages using the Griess assay

Schmölz L, Wallert M, Lorkowski S

Journal of Immunological Methods (2017); 449C, 68-70

DOI: 10.1016/j.jim.2017.06.012

Akzeptiert am 30. Juni 2017

Beitrag zum Manuskript

- Erstellung des Konzeptes
- Optimierung des Griess-Assays
- Durchführung von Griess-Assays
- Datenanalyse
- Evaluierung der Daten
- Erstellung des Manuskriptes

Eigenanteil am Manuskript: 80 %

Hauptaussagen des Manuskriptes 2

Zur Charakterisierung von Naturstoffen bezüglich ihres antiinflammatorischen Potentials ist die Anwendung des Griess-Assays zur Messung von NO verbreitet. Dies ist in Zellkulturüberständen häufig anspruchsvoll. Daher optimiert die Arbeit das Inkubationsregime für eine effektive Induktion der NO-Produktion, um die antiinflammatorischen Effekte der Testsubstanzen (LCM) messen zu können. Dabei spielen nicht nur die adäquate Zellkultur und der zeitliche Verlauf der Inkubation eine Rolle, sondern insbesondere die Art der Substanzapplikation ist von ausschlaggebender Bedeutung. So ist eine mehrmalige Applikation der Testsubstanz (vor und zeitgleich zur LPS-Gabe) optimal. Diese Arbeit zeigt das Vorgehen für eine systematische Optimierung auf und kann von Dritten als Ausgangspunkt für eigene Optimierungsarbeiten genutzt werden.

5.3 Manuskript 3 (veröffentlicht)

α-Tocopherol long-chain metabolite α-13′-COOH affects the inflammatory response of lipopolysaccharide-activated murine RAW264.7 macrophages

Wallert M, Schmölz L, Glei M, Krauth V, Werz O, Birringer M, Lorkowski S

Molecular Nutrition & Food Research (2015); 59, 1524-1534

DOI 10.1002/mnfr.201400737

Akzeptiert am 13. April 2015

Beitrag zum Manuskript

- Etablierung des NFκB-Western-Blots (*nuclear factor kappa-light-chain-enhancer of activated B-cells*, NFκB)
- Durchführung der NFκB-Western-Blots
- Datenanalyse
- Evaluierung der Daten
- Kritische Revision des Manuskriptes

Eigenanteil am Manuskript: 20 %

Hauptaussagen des Manuskriptes 3

Diese Originalarbeit zeigt die antiinflammatorische Kapazität von α-13′-COOH in murinen, LPS-stimulierten Makrophagen. Dafür werden mehrere pro- und antiinflammatorische Gene und Proteine mittels RT-qPCR und Western-Blot untersucht und auf funktioneller Ebene Mediatoren mittels ELISA (*enzyme linked immunosorbent assay*) vermessen. Die LPS-induzierte iNos- und Cox2-Expression wird dabei sowohl auf mRNA- als auch auf Proteinebene von α-13′-COOH effektiver gehemmt als durch α-TOH. Das gleiche Bild zeigt sich auch auf der funktionellen Ebene, so wird die NO- und Prostaglandinsynthese durch die LCM effektiver gehemmt. Daher kann vermutet werden, dass ein Teil der α-TOH-Effekte *in vivo* möglicherweise auf Effekte zurückzuführen sind, die durch α-LCM vermittelt werden.

5.4 Manuskript 4 (in Vorbereitung)

Long-chain metabolites of vitamin E: interference with lipotoxicity via lipid droplet associated protein PLIN2

Schmölz L, Schubert M, Kirschner J, Kluge S, Galli F, Birringer M, Wallert M, Lorkowski S

Veröffentlichung geplant in *BBA Molecular and Cell Biology of Lipids*

Beitrag zum Manuskript

- Erstellung des Konzeptes
- Durchführung der Zellvitalitätsmessungen, RT-qPCR, Western-Blots, der Versuche am Durchflusszytometer, der Transfektionsversuche
- Datenanalyse
- Evaluierung der Daten
- Erstellung des Manuskriptes

Eigenanteil am Manuskript: 50 %

Hauptaussagen des Manuskriptes 4

Diese Originalarbeit untersucht die Regulation des lipidtropfenassoziierten Proteins PLIN2 und der Lipotoxizität durch die LCM und stellt einen Zusammenhang zwischen beiden her. Die LCM induzieren die Expression von PLIN2 auf mRNA- und Proteinebene und erhöhen die Speicherung der Neutrallipide. Ebenso reduzieren sie die Stearinsäure (SA)-induzierte Lipotoxizität in humanen THP-1-Makrophagen. Durch einen *Knockdown* von PLIN2 mittels siRNA (*small interfering RNA*) kann der LCM-Effekt auf die Lipotoxizität verringert werden, was darauf hindeutet, dass PLIN2 an der LCM-vermittelten Reduktion der SA-induzierten Lipotoxizität beteiligt ist.

5.5 Manuskript 5 (veröffentlicht)

Regulatory metabolites of vitamin E and their putative relevance for atherogenesis

Wallert M*, Schmölz L*, Galli F, Birringer M, Lorkowski S

Redox Biology (2014); 2:495-503

DOI: 10.1016/j.redox.2014.02.002

Akzeptiert am 11. Februar 2014

Beitrag zum Manuskript

- Erstellung des Konzeptes
- Recherche und Evaluierung der Daten
- Erstellung des Manuskriptes

Eigenanteil am Manuskript: 40 %

Hauptaussagen des Manuskriptes 5

Der Übersichtsartikel legt kurz einige Grundlagen zur Atherogenese dar, um dann auf die Erkenntnisse zur Wirkweise des Vitamin E in der Entstehung der Atherosklerose *in vitro* und *in vivo* einzugehen. Dabei werden die Diskrepanzen zwischen den beiden Untersuchungsebenen und eine kontroverse Rolle des Vitamin E in der Atherogenese deutlich. Der Metabolismus von Vitamin E wird kurz dargestellt und eine Übersicht über die bisherigen Forschungsergebnisse zu den LCM wird gegeben. Außerdem werden folgende weiterführende Hypothesen aufgestellt: Die LCM wirken in geringerer Konzentration über einen von α-TOH verschiedenen Mechanismus und sind damit eine neue Klasse regulatorischer Metabolite, die zu physiologischen und pathophysiologischen Prozessen beitragen könnten und möglicherweise die Diskrepanzen zwischen den Ergebnissen der *In-vitro*- und *In-vivo*-Studien erklären.

5.6 Manuskript 6 (veröffentlicht)

Complexity of vitamin E metabolism

Schmölz L, Birringer M, Lorkowski S, Wallert M

World Journal of Biological Chemistry (2016); 7(1): 14-43

DOI: 10.4331/wjbc.v7.i1.14

Akzeptiert am 16. Januar 2016

Beitrag zum Manuskript

- Erstellung des Konzeptes
- Recherche und Evaluierung der Daten
- Erstellung des Manuskriptes

Eigenanteil am Manuskript: 50 %

Hauptaussagen des Manuskriptes 6

Der Übersichtsartikel fasst die aktuelle Erkenntnislage zum Metabolismus von Vitamin E zusammen. Dabei werden die Grundlagen, wie die Bioverfügbarkeit von Vitamin E, die intestinale Absorption, der vaskuläre Transport und die Bedeutung von intrazellulären Bindeproteinen beschrieben. Im zentralen Fokus steht aber der Metabolismus von Vitamin E in der Leber. Dieser wird im Detail beschrieben, wobei der enzymatische Katabolismus, die Kompartimentierung sowie die Ausscheidung eine wichtige Rolle spielen. Ergänzt wird das Manuskript mit Informationen zu genetischen Polymorphismen und metabolischen Interaktionen des Vitamin-E-Metabolismus mit anderen Stoffwechselwegen.

Damit wird die Komplexität und Bedeutung des Vitamin-E-Metabolismus umfassend dargestellt und die Basis für weiterführende Arbeiten gelegt. Eine der zentralen Thesen des Manuskriptes ist bspw., dass die Analytik der langkettigen Vitamin-E-Metabolite ein besserer Parameter zur Bestimmung des Vitamin-E-Status ist, als die Messung des α-TOHs im Blut. Diese These muss in weiterführenden Studien belegt werden, deutet aber auf eine zentrale Bedeutung der langkettigen Vitamin-E-Metabolite hin.

Manuskript 7 (veröffentlicht)

Garcinoic acid: a promising bioactive natural product for better understanding the physiological functions of tocopherol metabolites

Kluge S, Schubert M, Schmölz L, Birringer M, Wallert M, Lorkowski S

Studies in Natural Products Chemistry (2016); 51:435-481

DOI: 10.1016/B978-0-444-63932-5.00009-7

Akzeptiert am 24.02.2016

Beitrag zum Manuskript

- Überarbeitung einiger Abbildungen
- Kritische Revision des Manuskriptes

Eigenanteil am Manuskript: 10 %

Hauptaussagen des Manuskriptes 7

Der Übersichtsartikel erläutert die Bedeutung von Naturstoffen für die Entwicklung von Medikamenten anhand der GA, welche aus der afrikanischen Bitternuss *Garcinia kola* isoliert werden kann. Über chemische Modifikationen können die LCM semisynthetisch aus der GA erzeugt werden. Darüber hinaus wird ein Konzept zur Ableitung und Übertragbarkeit biologischer Funktionen von Vorläuferstrukturen und Strukturähnlichen auf die Zielsubstanzen erläutert. In diesem Falle wird die Datenlage zur Bioaktivität von TOH, T3 und GA beschrieben und anschließend mit den bekannten Aspekten der Bioaktivität der LCM verglichen. Abschließend werden zentrale Fragen um die Bioaktivität der LCM gestellt und weiterführende Versuche zur Aufklärung der Fragen vorgeschlagen.

6 Manuskripte

6.1 Manuskript 1

Structure-function relationship studies *in vitro* reveal distinct and specific effects of long-chain metabolites of vitamin E

Running Title: Structure-dependent effects of vitamin E metabolites

Lisa Schmölz [1, 2], Maria Wallert [1, 2, 3], Nicolò Rozzino [4], Andrea Cignarella [5], Francesco Galli [6], Michael Glei [2, 7], Oliver Werz [8], Andreas Koeberle [8], Marc Birringer [9], Stefan Lorkowski [1,2,*]

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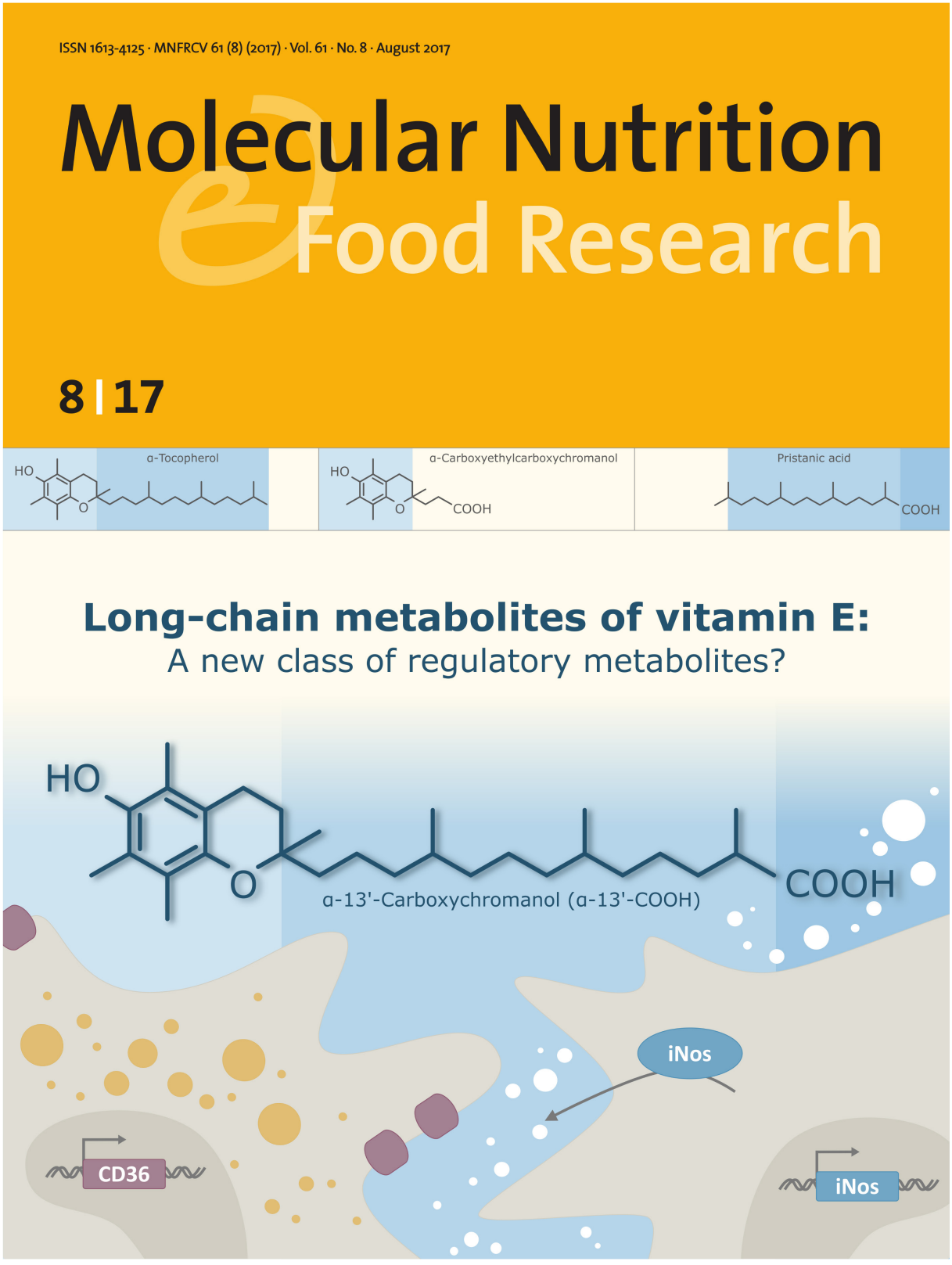
Keywords:

α-13'-OH, α-13'-hydroxychromanol, α-13'-COOH, α-13'-carboxychromanol, long-chain metabolites of vitamin E

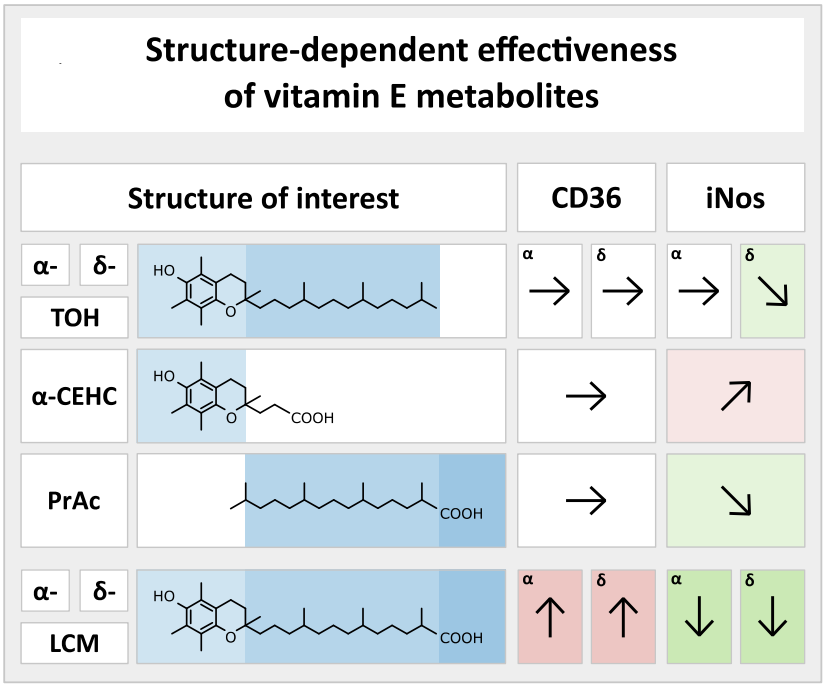
List of nonstandard abbreviations:

α-13'-OH, α-13'-hydroxychromanol, **α-13'-COOH,** α-13'-carboxychromanol, **α-CEHC,** α-carboxyethylhydroxychromanol, **CD36,** cluster of differentiation 36, **δ-13'-OH,** δ-13'-hydroxychromanol, **δ-13'-COOH,** δ-13'-carboxychromanol, **iNos,** inducible nitric oxide synthase, **LCM,** long-chain metabolites, **PrAc,** pristanic acid, **SCM,** short-chain metabolites, **TOH,** tocopherol.

Cover


ISSN 1613-4125 · MNFRCV 61 (8) (2017) · Vol. 61 · No. 8 · August 2017
Molecular Nutrition
Food Research
8 | 17
α-Tocopherol
α-Carboxyethylcarboxychromanol
Pristanic acid
Long-chain metabolites of vitamin E:
A new class of regulatory metabolites?
α-13'-Carboxychromanol (α-13'-COOH)
iNos
CD36
iNos

Graphical Abstract



Cytochrome-dependent metabolism of vitamin E initially forms long-chain metabolites (LCM) 13'-hydroxychromanols (13'-OH) and 13'-carboxychromanols (13'-COOH). A structure-activity relationship study using α- and δ-tocopherol, their 13'-LCM and representatives of their substructures (α-carboxyethylhydroxychromanol and pristanic acid) was performed to unravel structural elements required for biological activity. The LCM effects depend on the presence of the chromanol system and the modification of the side-chain but not on the substitution pattern of the chromanol ring. It can be concluded that for effects the entire LCM molecule is needed and that the effects are specific.

Abstract

Scope: Cytochrome-dependent metabolism of vitamin E initially forms the long-chain metabolites (LCM) 13'-hydroxychromanols (13'-OH) and 13'-carboxychromanols (13'-COOH), which occur in human blood. Little is known about their biological functions.

Material and Results: A structure-activity relationship study using α- and δ-tocopherol (TOH), their LCM (α-13'-OH, δ-13'-OH, α-13'-COOH and δ-13'-COOH) and representatives of their substructures (α-carboxyethylhydroxychromanol and pristanic acid) was performed to unravel critical structural elements of the LCM for biological activity. Prominent effects were mediated by α- and δ-LCM, as scavenger receptor cluster of differentiation 36 (CD36) expression was induced in human THP-1 macrophages and lipopolysaccharide-–stimulated inducible nitric oxide synthase (iNos) expression was inhibited in murine RAW264.7 macrophages, while the other molecules were less or not effective.

Conclusion: The LCM effects depend on the presence of the chromanol ring system and on the modification of the side-chain but not on the substitution pattern of the chromanol ring. Therefore, it can be concluded that for mediation of effects by LCM the entire molecule is needed and that the effects are specific. We propose the LCM of the micronutrient vitamin E as a new class of regulatory metabolites, but further studies are needed to corroborate this hypothesis.

Introduction

Vitamin E was discovered in 1922 by Evans and Bishop [1]. Despite its discovery almost 100 years ago, the physiological and molecular modes of action are still not fully unraveled. Vitamin E has been extensively studied in the field of redox biology [2], but its role in the prevention of diseases, such as atherosclerosis, is still unclear; results obtained *in vivo* and *in vitro* are often contradictory [3]. The principle hepatic metabolism of vitamin E is established and rather complex [4]. It is known that the hepatic metabolism, as shown in Figure 1, starts with an oxidation of the aliphatic side-chain by the cytochrome P (CYP) 450-dependent enzymes CYP4F2 and/or CYP3A4 [5, 6], forming the first physiological long-chain metabolites (LCM) 13'-hydroxychromanol (13'-OH) and 13'-carboxychromanol (13'-COOH), which also circulate within the blood of humans and may exert systemic effects [7-9]. Further catabolism of the LCM shortens the side-chain, leading to intermediate-chain metabolites (ICM) and finally to water soluble short-chain metabolites (SCM, carboxyethylhydroxychromanols, CEHC) [10]. At present, knowledge about the physiological function and importance of the LCM is limited [7, 8, 11-16].

Some of the physiological effects known for the LCM cover two key players of atherogenesis: the scavenger receptor cluster of differentiation 36 (CD36), whose expression is induced by the α-LCM [7], and the inducible nitric oxide synthase (iNos), whose lipopolysaccharide (LPS)-induced expression is diminished by the α-LCM [8, 11]. Both contribute to atherogenesis by macrophage foam cell formation [17] and via inflammatory processes.

To confirm the concept of distinct and specific LCM signaling, a comprehensive study on the contribution of molecular substructures of the LCM (Figure 2) was conducted. Their effects on α-LCM target genes, namely CD36 and iNos, were analyzed. Whether the presence of the chromanol ring system (approach I.a) is important, was tested using pristanic acid (PrAc), a branched-chain fatty acid with the same length as the side-chain of the LCM. The impact of the substitution of the chromanol ring system (approach I.b) was tested by using α- and δ-TOH and the respective α- and δ-LCM (*i.e.* α- and δ-13'-OH, and α- and δ-13'-COOH). The relevance of the side-chain (approach II.a) was evaluated through α-CEHC, the chain-shortened end-product of vitamin E catabolism. The influence of the side-chain modification (approach II.b) was assessed by comparing the effects of the precursors and the different LCM (TOH *vs.* 13'-OH *vs.* 13'-COOH). A similar approach has been conducted previously with vitamin E succinate to unravel structure-activity relationships [18].

1 Materials and Methods

Chemicals

If not indicated otherwise, chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), Fisher Scientific (Schwerte, Germany) or Merck Millipore (Darmstadt, Germany).

Cell culture

Human THP-1 monocytes and macrophages

THP-1 monocytes (ATCC, Manassas, VA), cultivated in RPMI-1640 supplemented with 10% (v/v) FBS and 0.1 mg/mL penicillin/streptomycin/L-glutamine [19], were differentiated into macrophages using 100 ng/mL phorbol-12-myristate-13-acetate (PMA) and 50 µM β-mercaptoethanol [20]. Macrophages were incubated as indicated in the figures and harvested as described below.

Murine RAW264.7 macrophages

RAW264.7 macrophages (ATCC) were cultivated in DMEM high-glucose (4.5 g/l) medium supplemented with 10% (v/v) FBS and 0.1 mg/mL penicillin/streptomycin/L-glutamine mixture and splitting was performed as described earlier [11]. Cells were incubated as indicated in the figures and harvested as described below.

Cytotoxicity and concentrations for cell culture studies

Cytotoxicity of LCM was assessed by sulforhodamine B assay as described earlier [7]. No detectable cytotoxicity was mediated by α-TOH, α-13'-OH, δ-TOH and δ-13'-OH up to 100 µM in THP-1 macrophages. The EC_{50} value for α-13'-COOH was 7.4 ± 1.5 µM [11] and 11.1 ± 4.0 µM for δ-13'-COOH. The concentration of the compounds was determined in pure ethanol via absorption. Wavelengths and attenuation coefficients used were 292 nm and ε = 3060 for α-13'-OH and α-13'-COOH, and 298 nm and ε = 3648 for δ-13'-OH and δ-13'-COOH.

RNA isolation and cDNA synthesis

Total RNA was isolated using Qiagen RNeasy Mini kit (Hilden, Germany) as described [21]. cDNA synthesis was performed using Revert Aid First strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and 500 ng/µL oligo-dT primers as described [22].

Quantitative real-time RT-PCR (RT-qPCR)

RT-qPCR was run on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) as described [22]. Primers (CD36, RPL37A, iNos, Ppib, Supplementary Table S1) were purchased from Invitrogen (Karlsruhe, Germany). PCR results were analyzed using the LightCycler software version 1.5.0.39.

Flow cytometry

After incubation cells were detached with Accutase I solution and analyzed using an Attune NxT Flow Cytometer. Anti-human FITC-labeled CD36 antibody (Beckman Coulter, Krefeld, Germany) was added at a final concentration of 5 µg/mL for 30 min at 4 °C. Data were analyzed with Attune NxT Flow Cytometer software version 2.2.0.8543.

Immunoblotting

Cells were harvested using a non-denaturing buffer and processed for Western blotting. Proteins were separated by SDS-PAGE and transferred to PVDF membrane (VWR, Darmstadt, Germany). Primary antibody against iNos (mouse anti-iNos clone 6, 1:2000) and against α-tubulin (mouse anti-α-tubulin clone B-5-1-2, 1:5000) were from BD Biosciences (Heidelberg, Germany) and Sigma-Aldrich, respectively. Secondary antibodies (rabbit anti-mouse labeled with horseradish peroxidase, 1:5000) from DAKO (Hamburg, Germany) were used. SignalBoost™ Immunreaction Enhancer Kit (Calbiochem, Darmstadt, Germany) was used for iNos.

Nitric oxide detection via Griess assay

RAW264.7 macrophages were incubated with test compounds for 4 h followed by another 20 h combined incubation with 100 ng/mL LPS as described earlier [23]. Supernatants were transferred to a 96-well plate, mixed with water and Griess reagent and incubated in the dark for 30 min. Nitrite concentration was measured at 544 nm with a FLUOstar omega microplate reader (BMG Labtech, Ortenberg, Germany). Analyses were performed using MARS data analysis software version 2.41. The calculation was referred to a standard dilution row of $NaNO_2$.

Isolation of garcinoic acid and semisynthesis of α- and δ-LCM

Garcinia kola seeds were a gift from AnalytiCon Discovery (Potsdam, Germany). Isolation of garcinoic acid from the African bitter nut *Garcinia kola* and syntheses of the LCM were performed as described [12, 24] and visualized in Supplemental Figure S1. Purity of all LCM was higher than 95%, as confirmed by HPLC-MS.

Statistics

Data are presented either as means ± standard deviation or as means ± standard error of the mean (SEM) of independent experiments as indicated. To test for statistical significance, paired Student's t-tests were performed using Microsoft Excel 2010 for data shown in Figures 3 and 5. The statistical evaluation of the data shown in Figure 4 was performed in R using a multiple pairwise t-test with Holm adjustment. For this, the data for each functional level (mRNA, protein and nitric oxide release) were tested within the α-forms and δ-forms.

2 Results

While there is convincing evidence for the systemic availability of the SCM of vitamin E in humans [25, 26], there was a lack of studies to demonstrate this for the LCM. Our group first measured α-13'-OH [8] and α-13'-COOH [7] in serum of volunteers using an unbiased LC-MS/MS method [9]. The serum concentrations of these LCM were recently reported to be about 1.2 nM and 0.8 nM at baseline and 2.4 nM and 8 nM after one week of supplementation with 1000 IU *RRR*-α-TOH, respectively [9]. This provided evidence for the systemic availability of

the LCM and for the possibility to markedly influence circulating and possibly tissue levels of these metabolites by the intake of the vitamin precursor.

Expression of CD36 in THP-1 macrophages is induced by the LCM but not by α-CEHC or PrAc

To evaluate the potential of α- and δ-LCM to prevent foam cell formation we used THP-1 macrophages incubated with α- or δ-TOH (100 µM) or their respective α- or δ-LCM (α- or δ-13'-OH: 10 µM; α- or δ-13'-COOH: 5 µM), PrAc (5 and 10 µM) or α-CEHC (5 and 10 µM). α- and δ-TOH modified CD36 expression neither on mRNA nor protein level (Figure 3A-D). In contrast, the α-LCM induced the expression of CD36 by 2.5-fold and 3.6-fold ($p < 0.01$) on mRNA and by 0.11-fold and 0.29-fold ($p < 0.05$) on protein level for α-13'-OH and α-13'-COOH, respectively, as compared to unstimulated control. Similarly, the δ-LCM induced expression of CD36 by 6.6-fold and 4.3-fold ($p < 0.01$) on mRNA and by 0.34-fold and 0.29-fold ($p < 0.05$) on protein level for δ-13'-OH and δ-13'-COOH, respectively. α-CEHC and PrAc had no effect on CD36 expression at any concentration tested (Figure 3E-H).

Lipopolysaccharide-induced iNos expression and nitric oxide formation in RAW264.7 macrophages are reduced by the LCM

RAW264.7 macrophages were incubated with 20 µM α- or δ-TOH (optimized concentration suitable for Griess assay [11]), 10 µM α- or δ-13'-OH, or 5 µM α- or δ-13'-COOH for 4 h prior to co-incubation with 100 ng/mL lipopolysaccharide (LPS) and the respective compound as described recently to be the optimal set-up [23]. In all experiments, the incubation of cells with LPS resulted in a significant increase of iNos mRNA or protein expression and nitric oxide release ($p < 0.001$, Figure 4). While α-TOH did not influence the LPS-induced expression of iNos, the release of nitric oxide was slightly induced by 14% (Figure 4A-C). α-13'-OH (Figure 4G-I) inhibited the LPS-induced iNos expression on the mRNA level (29% inhibition, $p < 0.01$, Figure 4G) and the protein level (40% inhibition, $p < 0.05$, Figure 4H). Accordingly, α-13'-OH blocked the release of nitric oxide by 56% ($p < 0.001$, Figure 4I). Even more pronounced effects were observed when α-13'-COOH (Figure 4M-O) was used. An inhibition of the LPS-induced iNos mRNA expression of 97% ($p < 0.001$, Figure 4M) and a full block of protein expression ($p < 0.001$, Figure 4N) in parallel to an inhibition of nitric oxide release of 78% ($p < 0.001$, Figure 4O) were measured following α-13'-COOH treatment.

The δ-forms revealed the same effects, except for δ-TOH (Figure 4D-F), which also inhibited the LPS-induced effects on all levels tested (by 36% to 60%, $p < 0.05$ at least, Figure 4D-F). Again, the effect increased from δ-TOH over δ-13'-OH (Figure 4J-L) to δ-13'-COOH (Figure 4P-R), as the LPS-induced effects were inhibited by δ-13'-OH by 49% to 67% ($p < 0.001$, Figure 4J-L). δ-13'-COOH was the most potent δ-form (Figure 4P-R) and reduced the LPS-induced effects by 52% to 91% ($p < 0.001$).

PrAc and α-CEHC affect LPS-induced iNos expression and nitric oxide production in RAW264.7 macrophages

To reflect the concentrations tested in the aforementioned experiments, 5 µM or 10 µM PrAc (Figure 5A-C) and α-CEHC (Figure 5D-F) were used, and analyses on mRNA (Figure 5A and 5D) and protein level (Figure 5B and 5E) or nitric oxide release (Figure 5C and 5F) were performed. In all experiments, the LPS stimulus resulted in a significant induction of iNos

expression and nitric oxide release (at least $p < 0.01$). Pristanic acid significantly inhibited the LPS-induced expression of iNos in a concentration-dependent manner on the mRNA and protein level (20% to 64%, $p < 0.05$ at least; Figures 5A and 5B). Furthermore, PrAc reduced the LPS-induced release of nitric oxide by 33% to 49% ($p < 0.05$ at least; Figure 5C). The SCM α-CEHC had no effect on the LPS-induced expression of iNos mRNA (Figure 5D), whereas expression of iNos protein and LPS-induced release of nitric oxide was significantly induced at both concentrations used (19% to 47%, $p < 0.05$; Figures 5E and 5F).

3 Discussion and Conclusions

Research on the molecular mechanisms of vitamin E, even in the context of atherosclerosis, has gone out of fashion and abandoned by the funding strategies of institutional and private organizations; that was the consequence of the absence of evidence on clinical endpoints assessed in large randomized trials carried out in the last two decades (reviewed in [27]). In this context, a renewed interest in this vitamin emerged from the investigation of its metabolites, and particularly the LCM, that some of us first synthesized from the plant analogue garcinoic acid and characterized as bioactive molecules *in vitro* [28]. Since then, we and other groups confirmed such biological properties of the LCM in a series of *in vitro* and *in vivo* studies (reviewed in [4]). To shed new light on this topic, we focused on the physiological hepatic metabolites of TOH, the LCM 13'-OH and 13'-COOH (see Figure 1 for details).

To get deeper insights into the molecular details, we conducted a comparative study on the contribution of substructures of the LCM molecule to their regulatory effects. We compared the effects of the precursor α-TOH and δ-TOH and their respective LCM (α-13'-OH, α-13'-COOH, δ-13'-OH, δ-13'-COOH) with compounds representing either the chromanol ring system (*i.e.* α-CEHC) or the oxidized aliphatic side-chain (*i.e.* PrAc; see Figure 2 for details).

We concentrated on two proteins, whose expression has been previously shown to be regulated by the α-LCM, *i.e.* the scavenger receptor CD36 [17] and the inflammatory enzyme iNos [29]. To the best of our knowledge, this is the first study to describe the regulatory effects of the δ-LCM on CD36 expression, iNos expression and nitric oxide release. Further on, a systematic comparison of the effects of α- to δ-LCM has not been described.

For our studies, we used cells that are unable to metabolize vitamin E (unpublished results), namely human THP-1 and murine RAW264.7 macrophages. In order to be close to the human system, we used human THP-1 macrophages for studies related to cellular lipid homeostasis. As these cells are no appropriate model to study the inflammatory action of iNOS due to a lack of the functional protein [30], we used murine RAW264.7 macrophages, which are a more suitable model for studying the anti-inflammatory potential of test compounds [31].

In the present study, α-TOH and δ-TOH did not regulate the expression of CD36 (Figure 3), but their respective LCM clearly induced CD36 mRNA and protein expression, even at lower

concentrations than those of their precursor (13'-COOH and 13'-OH (5 to 10 µM) vs. TOH (100 µM)). To our knowledge, there is no information available about the regulatory capacity of δ-TOH regarding CD36 expression. In contrast, the results for α-TOH vary, depending on the experimental design, ranging between no significant effects [2, 32-34] and inhibitory effects [7, 35-38]. In two studies [7, 38], which are comparable to our studies, a clear and significant downregulation of CD36 mRNA expression was seen under α-TOH treatment. Comparing the effect sizes, it must be admitted that the regulation in this study is comparable to the aforementioned, but the statistical test revealed no significance. Therefore, we conclude that the effects for α-TOH in our study on CD36 expression are in line with results from previous studies. Wallert et al. [7] focused on the effects of the α-LCM on CD36 expression and described results comparable to the ones presented here. Although the LCM-mediated increase in CD36 in macrophages suggests an augmented foam cell formation, the authors found a reduced uptake of oxLDL likely due to reduced phagocytosis. However, the δ-LCM have not been studied in this context thus far. Interestingly, the effects mediated by the δ-LCM are comparable to those of the α-LCM.

The molecules representing substructures of the LCM, α-CEHC and PrAc, failed to regulate CD36 expression in the concentration range tested. Their influence on CD36 expression has not been described yet. Our results clearly show that neither the precursors nor the molecules representing substructures of the LCM share the effects of the LCM.

While α-TOH regulated neither iNos expression nor nitric oxide release, δ-TOH inhibited the LPS-mediated induction (Figure 4). The same holds true for all α- and δ-LCM. Interestingly, α- and δ-13'-COOH were generally more efficient than α- and δ-13'-OH, even at lower concentrations. In the literature, the regulation of iNos protein expression after LPS challenge by α-TOH in murine macrophages has been investigated in several studies, and the outcome varied between no effects [8, 11, 39, 40] and inhibitory effects [41, 42]. The latter reported a moderate inhibitory effect of about 25% on the LPS-induced expression of iNos by α-TOH, in a concentration range, which is comparable to our study. Furthermore, an approximate 30% reduction of the LPS-induced nitric oxide release by α-TOH has been shown [40, 41], whereas other studies found no effect [8, 11, 39, 42-44]. These discrepancies may occur due to differences in the experimental setup. However, the effects of TOH are more inconsistent than for the LCM and in all cases the effects of TOH were significantly smaller than those of the LCM, even at remarkably higher concentrations.

The effects of δ-TOH on the LPS-induced expression of iNos and the release of nitric oxide are unknown. However, studies on prostaglandin (PG)E_2 release or COX-2 expression in LPS-stimulated cells showed that non-α-TOH forms exert effects in a distinct and more effective manner than α-TOH [13, 45]. The findings on α- and δ-TOH in our study are in line with these findings.

The knowledge about biological effects of the LCM is limited, however, two previous studies conducted by our group found that α-13'-OH [8] and α-13'-COOH [11] efficiently block the LPS-induced expression of iNos and the release of nitric oxide, which represents our results shown here. To the best of our knowledge, nothing is known about the regulatory effects of the δ-LCM in this context. We are therefore the first to describe the inhibitory potential of δ-LCM on the

LPS-induced expression of iNos or the release of nitric oxide. Jiang et al. found a reduced COX-2 activity by δ-9'-COOH and δ-13'-COOH in cells pre-incubated with arachidonic acid [13]. Taken together, we confirm an anti-inflammatory potential of δ-LCM that surely deserves further investigation.

α-CEHC and PrAc showed opposing effects on the expression of iNos and the release of nitric oxide (Figure 5). While PrAc inhibited the LPS-induced effects in all parameters tested, α-CEHC increased the LPS-induced expression of iNos protein and the release of nitric oxide. The only study analyzing the regulatory effects of α-CEHC on iNos expression and nitric oxide release reported an inhibition at both levels in EOC-20 cells [44]. However, the results cannot be compared directly, as the resident murine microglial EOC-20 macrophages may react different than the murine leukemia RAW264.7 macrophages. Furthermore, the concentrations used were much higher (20 to 100 µM) as compared to those used in our study.

PrAc is a branched-chain fatty acid and its effects on the LPS-induced expression of iNos have not been studied thus far; however, it is known that its metabolic precursor phytanic acid induces iNos expression at 100 µM in vascular smooth muscle cells [46]. The effects of PrAc remained speculative. Although we showed that PrAc significantly reduced iNos expression and nitric oxide formation, the effects were smaller than that of the structurally related LCM.

The concentration, and also the mode of application, of our compounds at the site of action needs to be evaluated critically. First, the concentration used for the TOHs (100 µM or 20 µM) is supra-physiological but comparable to already published studies [36-38, 47, 48]. Pre-tests using lower concentrations revealed no significant regulation of CD36 in our hands. Second, the mode of application has not been evaluated. The dissolution of our compounds in DMSO and cell culture medium is far off from being physiological. Further studies are required to better mirror physiological conditions. Third, the intracellular bioavailability of the LCM depends on the efficiency of their uptake. Preliminary data of our group indicate an efficient uptake of the α-LCM in the absence of serum and within the incubation times used in our study; the other LCM have not yet been studied. In future studies, we must investigate which concentrations and modes of application match the physiological situation.

Structure-activity relationships, similar to the experiments reported here, have been performed using tocopheryl succinate [18, 49], and other synthetic forms of vitamin E [50] assessed as anti-cancer agents, which led to the conclusion that the substructures of tocopherol (chromanol ring system, hydroxyl group at the ring system and the side-chain) exhibit different biological activities (signaling, antioxidant and membrane docking domain, respectively) [49]. In line with these results for TOH, we conclude here that the full structure of the LCM, consisting of a chromanol ring system (almost independent of its methylation pattern (α- or δ-LCM)), an aliphatic side-chain, and an oxidative modification (the terminal hydroxyl- or carboxyl-groups) are required to exert the distinct effects of the LCM. Although some substructure-representing compounds regulate the effects in a similar fashion as the LCM, the concentrations required were significantly higher or the efficiencies were significantly smaller.

Overall, our findings presented here allow the conclusion that the LCM exert specific and distinct effects. These are mediated by the interaction with molecular targets that require the

full LCM molecule to occur, thus suggesting the presence of a receptor or a specific signaling pathway of the LCM. Based on the data available there is increasing evidence that the LCM comprise an interesting new class of regulatory metabolites of vitamin E. Further studies are required to prove this hypothesis and to unravel the physiological importance of the LCM.

Author contributions

L.S., M.W. and N.R. performed the experiments. L.S., M.W. and S.L. designed the study. M.W., M.B. and S.L. supervised the project. L.S. wrote the manuscript. M.W., A.C., F.G., M.G., O.W., A.K. and S.L. carefully read and evaluated the manuscript and discussed the results.

Acknowledgements

The work of S.L. is supported by grants from the Federal Ministry of Education and Research (01EA1411A), the Deutsche Forschungsgemeinschaft (DFG; RTG 1715) and the German Ministry of Economics and Technology (AiF 16642 BR) via AiF (German Federation of Industrial Research Associations) and FEI (Research Association of the German Food Industry) and by the Free State of Thuringia and the European Social Fund (2016 FGR 0045). The work of L.S. is supported by the Free State of Thuringia and the European Social Fund (2016 FGR 0045). The work of M.W. is also funded by the DFG (Wa 3836/1-1). We thank Alexander Maxones, Waltraud Scheiding and Maria Braun for their excellent technical assistance. We thank Markus Bernhardt-Römermann for this excellent support with the statistical evaluation of data. The work of O.W. is supported by the Deutsche Forschungsgemeinschaft (DFG; SFB1127 and FOR1406) and by the Free State of Thuringia and the European Social Fund (2016 FGR 0045).

Conflict of interest

The authors have declared no conflicts of interest.

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Figures

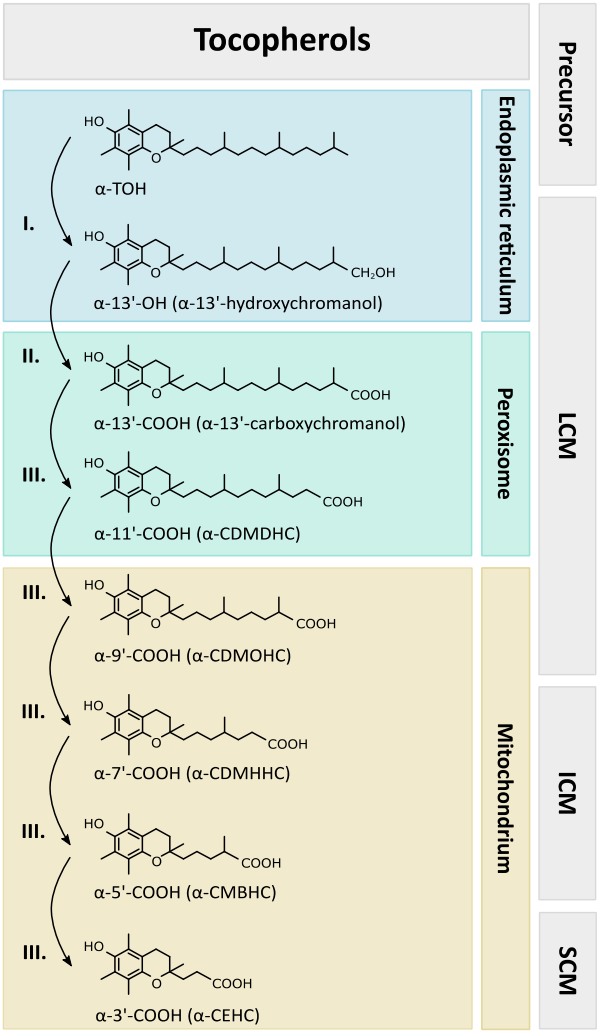

Figure 1
Schematic overview of the hepatic metabolism of vitamin E
The coordinated metabolism of vitamin E is an effective physiological pathway to protect tissues against excessive accumulation of particularly non-α-tocopherol (TOH) forms [4]. Initially, one cycle of CYP4F2/CYP3A4-dependent ω-hydroxylation (**I.**) forms the first physiological long-chain metabolite (LCM) 13'-OH. A subsequent ω-oxidation (**II.**) leads to 13'-COOH, which is followed by five cycles of subsequent β-oxidation (**III.**), forming the hydrophilic end-product carboxyethylhydroxychromanol (CEHC, short-chain metabolite (SCM)).

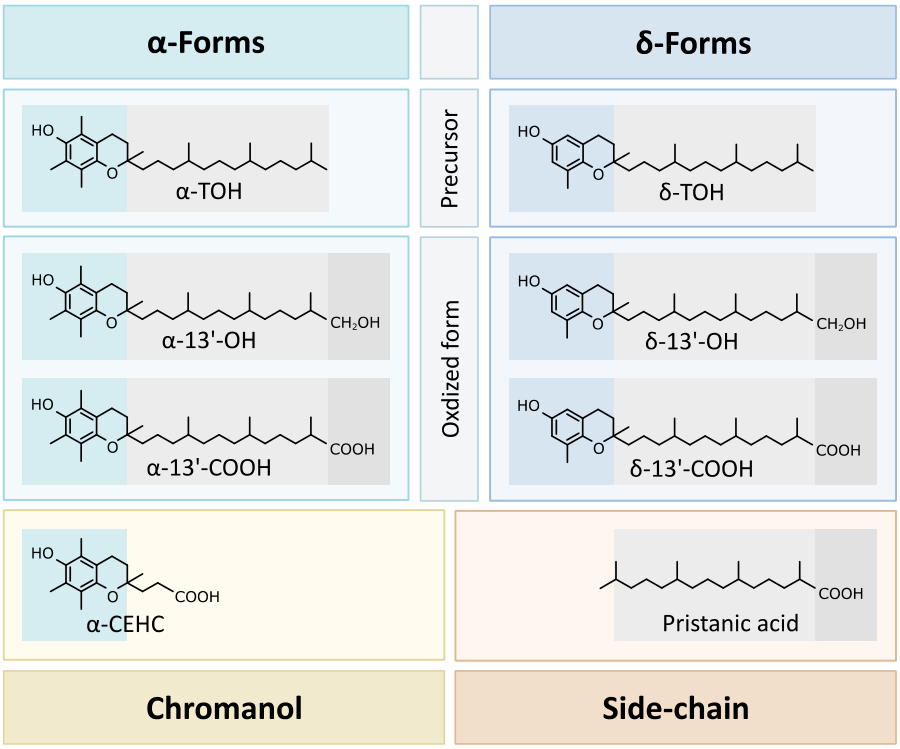

Figure 2

Schematic overview of the relevant structures used to unravel the structure-function relationships of the long-chain metabolites of vitamin E

The relevance of several structural features of the LCM, such as the modified side-chain and the substitution of the chromanol ring was tested using α- and δ-LCM (α-13'-OH, α-13'-COOH, δ-13'-OH, δ-13'-COOH) as well as their metabolic precursors α-TOH and δ-TOH. Their influence on the expression of CD36 and iNos was analyzed and compared to the effects of PrAc (similar to the side-chain of the LCM) and α-CEHC (representative of the chromanol ring system).

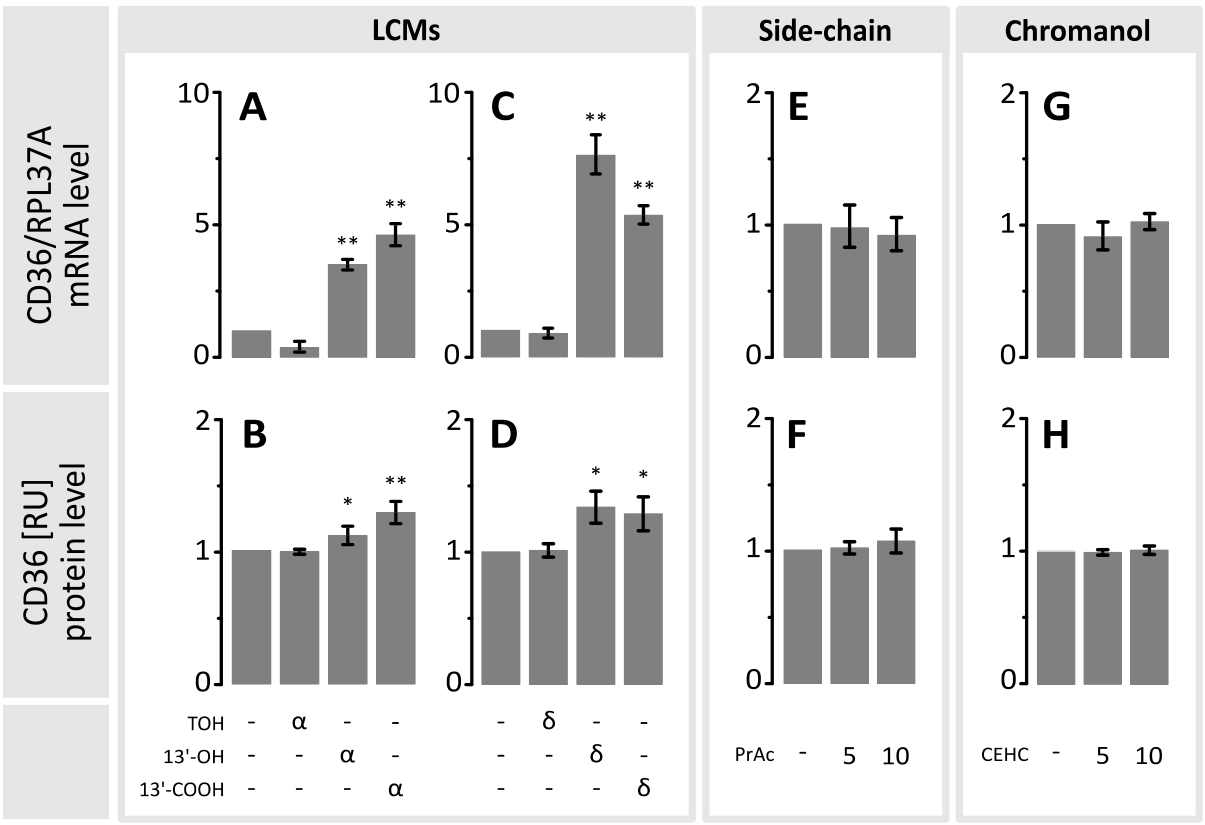

Figure 3

Expression of CD36 in human THP-1 macrophages is induced by LCM but not by α-CEHC or PrAc

Human THP-1 monocytes were differentiated and incubated for 24 h with α-TOH (100 µM) , α-13'-OH (10 µM) or α-13'-COOH (5 µM) **(A)** and **(B)**, δ-TOH (100 µM), δ-13'-OH (10 µM) or δ-13'-COOH (5 µM) **(C)** and **(D)**, PrAc (5 µM or 10 µM) **(E)** and **(F)**, and α-CEHC (5 µM or 10 µM) **(G)** and **(H)** for RT-qPCR analyses **(A), (C), (E)** and **(G)** or flow cytometric analyses **(B), (D), (F)** and **(H)**. Expression of CD36 was not regulated by α-TOH neither on the mRNA **(A)** nor on the protein **(B)** level, whereas α-13'-OH induced CD36 mRNA to 350% (SEM min 14%, SEM max 15%) and protein to 111% ± 6%. α-13'-COOH induced CD36 mRNA levels to 462% (SEM min 34%, SEM max 37%) and protein to 129% ± 7%. δ-TOH did not affect CD36 expression on the mRNA **(C)** or the protein **(D)** level. The LCM δ-13'-OH induced CD36 mRNA expression to 757% (SEM min 70%, SEM max 77%) and protein expression to 134% ± 12%, while δ-13'-COOH induced CD36 on the mRNA level by 532% (SEM min 33%, SEM max 36%) and on the protein level to 129% ± 13%. Neither PrAc nor α-CEHC were able to regulate CD36 expression at the concentrations tested. **(A)**, **(C)**, **(E)** and **(G)** mRNA expression levels were normalized to RPL37A mRNA expression. Expression of RPL37A remained unchanged under all treatments (not shown). Error bars display calculated maximum and minimum expression levels of mean expression levels of three independent biological experiments each measured once. **(B)**, **(D)**, **(F)** and **H)** Error bars display calculated maximum and minimum expression levels of four independent biological experiments each measured once. *, $p < 0.05$; **, $p < 0.01$ (*vs*. control).

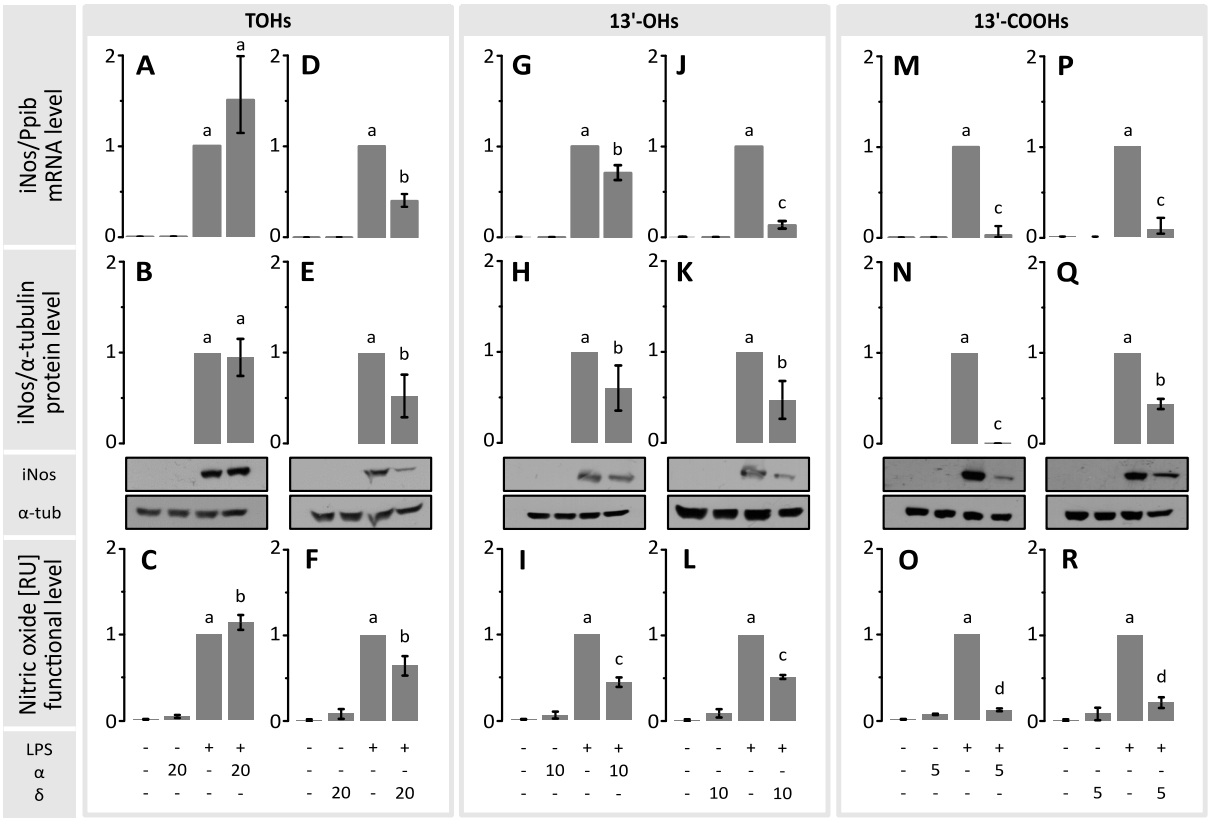

Figure 4

LPS-induced iNos expression and nitric oxide production is diminished by the LCM in RAW264.7 macrophages

Murine RAW264.7 macrophages were pre-incubated with α-TOH (20 µM) **(A)**, **(B)** and **(C)**, or δ-TOH (20 µM) **(D)**, **(E)** and **(F)**, or α-13'-OH (10 µM) **(G)**, **(H)** and **(I),** or δ-13'-OH (10 µM) **(J)**, **(K)** and **(L)**, or α-13'-COOH (5 µM) **(M)**, **(N)** and **(O)**, or δ-13'-COOH (5 µM) **(P)**, **(Q)** and **(R)** for 4 h followed by co-incubation of 100 ng/mL LPS and the respective compounds for further 20 h. Expression of iNos mRNA was analyzed by RT-qPCR **(A)**, **(D)**, **(G)**, **(J)**, **(M)** and **(P)**, iNos protein levels were quantified by Western blotting **(B)**, **(E)**, **(H)**, **(K)**, **(N)** and **(Q)**, and iNos activity was studied by measuring nitric oxide via Griess assay **(C)**, **(F)**, **(I)**, **(L)**, **(O)** and **(R)**. The bars in each subfigure represent from left to right the unstimulated control, the respective compound alone, LPS and co-incubation of the respective compound and LPS. The LPS-stimulated samples were defined as 1, and changes of all other samples were referred to the LPS-stimulated control. Neither LPS-induced expression of iNos mRNA **(A)** nor protein **(B)** was influenced by α-TOH, while nitric oxide production was slightly induced by 14% ± 9% **(C)**. δ-TOH reduced LPS-induced iNos expression significantly to 40% (SEM min 6%, SEM max 8%) on the mRNA level **(D)** and to 52% ± 24% on the protein level **(E)** and inhibited LPS-induced nitric oxide production to 64% ± 11% **(F)**. The α- and δ-LCM also reduced the LPS-induced expression of iNos and the production of nitric oxide significantly. α-13'-OH caused a significant reduction of LPS-induced expression of iNos to 71% (SEM min 8%, SEM max 9%) on the mRNA level **(G)**, to 60% ± 25% on the protein level **(H)** and nitric oxide production to 44% ± 6% **(I)**. In line with the findings for α-13'-OH, LPS-induced expression of iNos was also significantly diminished by δ-13'-OH to 13% (SEM min 4%, SEM max 5%) on the mRNA level **(J)** and to 47% ± 21% on the protein level **(K)**, and nitric oxide production was reduced to 51% ± 2% **(L)**. α-13'-COOH significantly reduced the LPS-induced expression of iNos to 3% (SEM min 2%, SEM max 10%) on the mRNA level **(M)** and blocked it completely to 0% on the protein level **(N)**, and nitric oxide production was inhibited to 12% ± 2% **(O)**. LPS-induced expression

of iNos was reduced significantly by δ-13'-COOH to 9% (SEM min 5%, SEM max 12%) on the mRNA level **(P)** and to 44% ± 6% on the protein level **(Q),** and nitric oxide production was attenuated down to 21% ± 6% **(R)**. **(A)**, **(D)**, **(G)**, **(J)**, **(M)**, and **(P)**: iNos mRNA expression levels were normalized to Ppib mRNA expression. Expression of Ppib changed during LPS treatment, but optical inspection of the cells under the light microscope and also the yield of extracted RNA was not influenced. However, the slight changes in the expression of the reference gene did not affect the principal findings. Error bars display calculated maximum and minimum expression levels of four independent biological experiments each measured once **(A)**, **(D)**, **(M)**, and **(P)** or twice **(G)** and **(J)**. **(B)**, **(E)**, **(H)**, **(K)**, **(N)** and **(Q)**: iNos protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions (only representative blots are shown). Images of Western blot analyses are shown that represent typical results. Mean expression levels of four **(B)**, **(E)**, **(H)** and **(K)** or five **(N)** and **(Q)** independent biological experiments each measured once are shown. **(C)**, **(F)**, **(I)**, **(L)**, **(O)** and **(R)**: Formation of nitric oxide was measured using Griess assay. Mean levels of three independent biological experiments are shown. Different characters indicate statistically valid differences between values.

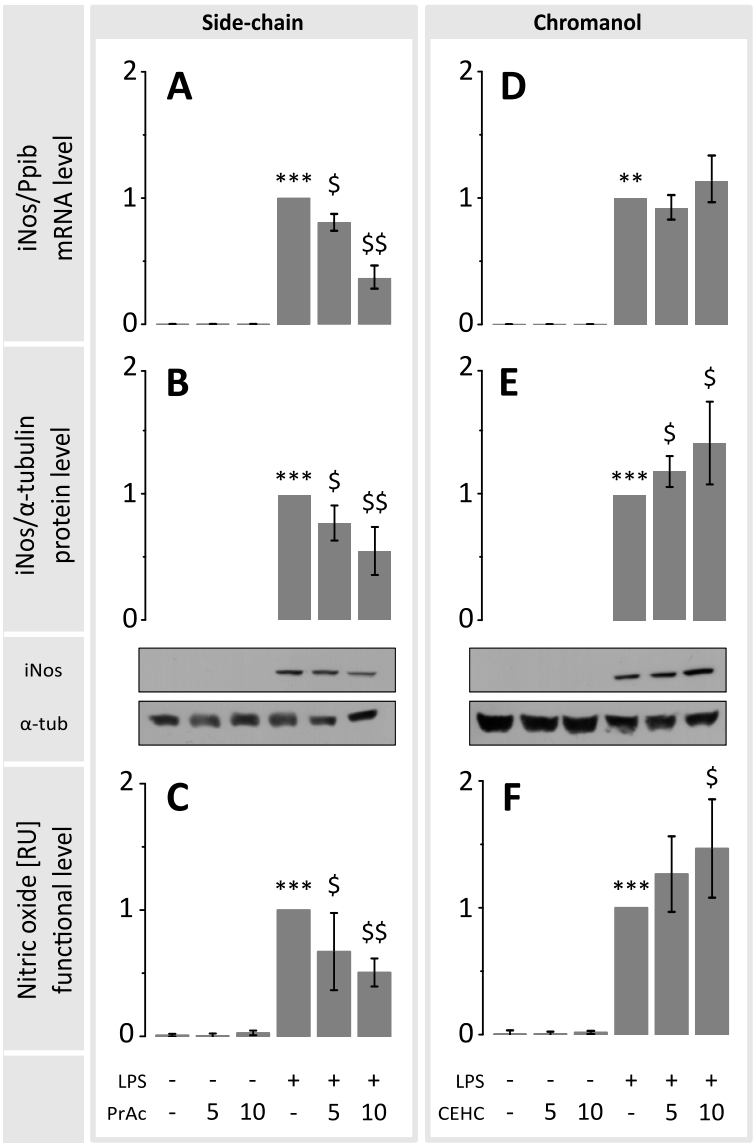

Figure 5

Lipopolysaccharide-induced iNos expression and nitric oxide production is induced by α-CEHC but reduced by PrAc in RAW264.7 macrophages

Murine RAW264.7 macrophages were pre-incubated with PrAc (5 µM or 10 µM) **(A)**, **(B)** and **(C)** or with α-CEHC (5 µM or 10 µM) **(D)**, **(E)** and **(F)** for 4 h followed by a co-incubation of 100 ng/mL LPS and the respective compounds for further 20 h. Expression of iNos mRNA was analyzed by RT-qPCR **(A)** and **(D)**, iNos protein levels were quantified by Western blotting **(B)** and **(E)**, and iNOS activity was studies by measuring nitric oxide via Griess assay **(C)** and **(F)**. The histogram bars in each panel represent from left to right: the unstimulated control, incubation with the respective compound in 5 µM and 10 µM, LPS stimulation alone and co-incubation of the respective compound and LPS. For all experiments, the LPS-stimulated sample was defined as 1. Changes of all other samples were referred to the LPS-stimulated control. The LPS-induced expression of iNos was significantly reduced by PrAc to 80% (SEM min 6%, SEM max 7%) at 5 µM or 36% (SEM min 8%, SEM max 10%) at 10 µM on the mRNA level **(A)**, and to 78% ± 14% for 5 µM and to 55% ± 19% for 10 µM on the protein level **(B)**, and nitric oxide production was diminished to 67% ± 31% for 5 µM and to 51% ± 11% for 10 µM **(C)**. The SCM α-CEHC showed no significant effect on LPS-induced iNos mRNA expression **(D)**, but iNos protein expression was significantly induced to 119% ± 12% for 5 µM and to 142% ± 33% for 10 µM **(E)**; LPS-induced nitric oxide production was significantly augmented at 10 µM to 147% ± 39% **(F)**. **(A)** and **(D)**: iNos mRNA expression levels were

normalized to Ppib mRNA expression. Expression of Ppib changed slightly during LPS treatment; however, optical inspection of the cells under the light microscope and also the yield of extracted RNA were not influenced obviously. The observed changes in the expression of the reference gene did not affect the principal findings. Error bars display calculated maximum and minimum expression levels of four independent biological experiments each measured once **(D)** or twice **(A)**. **(B)** and **(E)**: iNos protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions. Representative images of the Western blots for densitometry quantification are shown. Mean expression levels of five independent biological experiments each measured once are shown **(N)** and **(Q)**. **(C)** and **(F)**: Release of nitric oxide was measured using Griess assay. Mean expression levels of six independent biological experiments are given. **, $p < 0.01$, ***, $p < 0.001$ (*vs*. control); $^{\$}$, $p < 0.05$, $^{\$\$}$, $p < 0.01$, (*vs*. LPS treatment).

Supporting Material

Structure-function relationship studies *in vitro* reveal distinct and specific effects of long-chain metabolites of vitamin E

Running Title: Structure-dependent effects of vitamin E metabolites

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Table S1: PCR primers used in this study. In each case, forward and reverse primers are located in different exons.

mRNA	mRNA name	Origin	GenBank accession no.	Forward primer	Reverse primer	Amplicon size [bp]
CD36	Cluster of differentiation 36	Human	NM_001001548.2 NM_001001547.2 NM_000072.3 NM_001127443.1 NM_001127444.1	TCACTGCGACATGATTAATGGTACA	ACGTCGGATTCAAATACAGCATAGAT	126
RPL37A	Ribosomal protein L37a	Human	NM_000998	ATTGAAATCAGCCAGCACGC	AGGAACCACAGTGCCAGATCC	94
iNos/Nos2	Inducible nitric oxide synthase	Murine	NM_010927.3	GAGCGAGGAGCAGGTGGAA	CCATAGGAAAAGACTGCACCGA	90
Ppib	Peptidylprolyl isomerase B	Murine	NM_011149.2	AAACAGCAAGTTCCATCGTGTCAT	GAAGCGCTCACCATAGATGCTCT	103

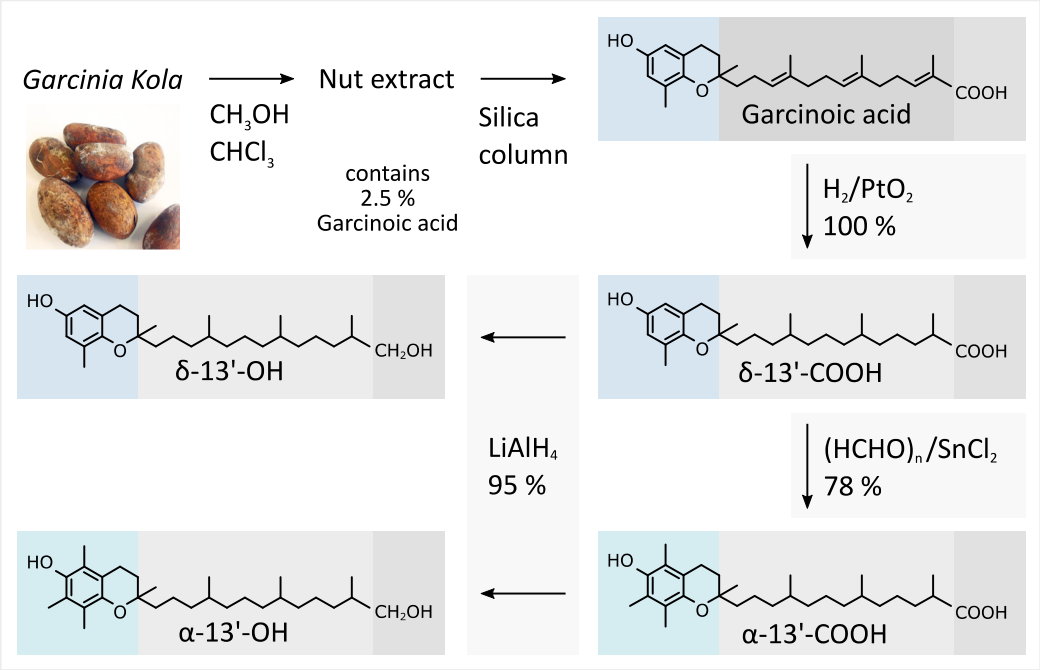

Figure S1
Isolation of garcinoic acid and semi-synthesis of α- and δ-LCM
The African bitter nut *Garcinia kola* was used to obtain pure garcinoic acid (δ-tocotrienoloic acid). Isolation of garcinoic acid and syntheses of the LCM were performed as described [12, 19].

6.2 Manuskript 2

Journal of Immunological Methods 449 (2017) 68–70

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

Optimized incubation regime for nitric oxide measurements in murine macrophages using the Griess assay

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ARTICLE INFO

Article history:
Received 1 June 2017
Received in revised form 30 June 2017
Accepted 30 June 2017
Available online 1 July 2017

Keywords:
Griess assay
Nitric oxide
Long-chain metabolites of α-tocopherol
α-13′-COOH
Incubation regime

ABSTRACT

The Griess assay is used to measure nitric oxide concentrations in liquid solutions after reaction into nitrite. The assay is challenging when applied to cell culture supernatants. During optimization, we focused on the anti-inflammatory potential of test compounds in murine RAW264.7 macrophages. This led to (i) the required inductivity of cells by lipopolysaccharide (LPS) and allowed (ii) the characterization of putative anti-inflammatory test compounds with high sensitivity. The modifications reported here prominently improved resolution and efficiency of the widely used Griess assay and are of broad interest for studies on the pharmacological modulation of macrophages activation.

1. Introduction

The use of the Griess assay to evaluate nitric oxide (NO) formation in different biological fluids has a longstanding history. It was first used to measure NO formation indirectly via nitrite concentrations in body fluids, namely urine and blood. It was soon realized that an optimization for each test matrix is necessary. However, today the Griess assay is also widely used to measure NO formation in cell culture supernatants. From our point of view, it is not surprising that even here an optimization of the assay is of great value. Nevertheless, no study describing a systematic optimization of the Griess assay for measurements of cell culture supernatants has been published so far.

Here, a comprehensive optimization scheme is presented, which leads to the most reliable results with high sensitivity when investigating putative anti-inflammatory test compounds. Our modifications prominently improved the resolution and efficiency of the widely used Griess assay.

In short, murine RAW264.7 macrophages were seeded and incubated with the compound of interest and lipopolysaccharide (LPS) for activating the oxidative burst. Upon LPS stimulus, the highly unstable NO molecule is released by the cells and rapidly converted to nitrite (Tsikas, 2007), which in turn is detectable by the Griess assay. To reliably evaluate the anti-inflammatory potential of test compounds the activation of RAW264.7 macrophages by LPS to a desired level above 20 μM NO is required. This self-set, experience-based threshold of 20 μM LPS induced NO release is in our hands a good marker, whether the macrophages were sufficiently activated, and allows in turn to reliably measure proposed anti-inflammatory effects of the test compounds. Thereby, an increased resolution of the Griess assay can be achieved, which allows the reliable characterization of test compounds with low anti-inflammatory potential.

2. Material and methods

2.1. Cell culture

Murine RAW264.7 macrophages were cultured in high glucose (4.5 g/l) Dulbeccos Modified Eagles Medium (DMEM, #D5796, Sigma-Aldrich) containing 10% (v/v) fetal bovine serum Superior (#S 0615, Biochrom, Germany) and 1% (v/v) L-glutamine-penicillin-streptomycin solution (#G1146, Sigma-Aldrich); for sub-culturing, cells were detached using a rubber policeman according to ATCC's instructions (ATCC, 2017). In addition, the culture medium was complemented with 30% medium from the previous cultivation passage as described (Wallert et al., 2015). The cells were seeded in 24-well plates (2×10^6 cells in 2 ml per well) and rested for 24 h to allow attachment to the well surface. While the Griess assay was performed in 96-well plates, the incubation of the RAW 264.7 cells was carried out in 24-well format to bypass the edge effect during incubation, which is known to be more prominent in smaller well formats (Lundholt et al., 2003). Incubation

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http://dx.doi.org/10.1016/j.jim.2017.06.012
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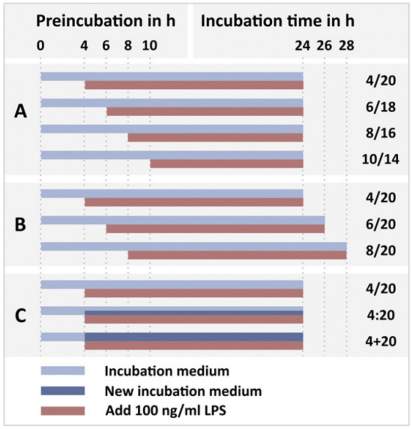

Fig. 1. Multistep approach for optimizing resolution and efficiency of the Griess assay. Murine RAW264.7 macrophages were incubated with test compounds and LPS. (A) A total incubation time of 24 h was split into pre-incubation and LPS challenge (4 h LCM plus 20 h LCM and LPS, 6 h plus 18 h, 8 h plus 16 h, and 10 h plus 14 h). (B) Next, the period of the LPS challenge was fixed to 20 h, but pre-incubation times varied (4 h plus 20 h, 6 h plus 20 h, and 8 h plus 20 h). (C) Finally, the application mode for the test compounds and LPS was changed. The incubation medium was either not replaced (indicated as 4/20), partly replaced (indicated as 4:20; 500 µl) or completely replaced (indicated as 4 + 20). This resulted in an increasing amount of test compound used (from 1-fold to 2-fold).

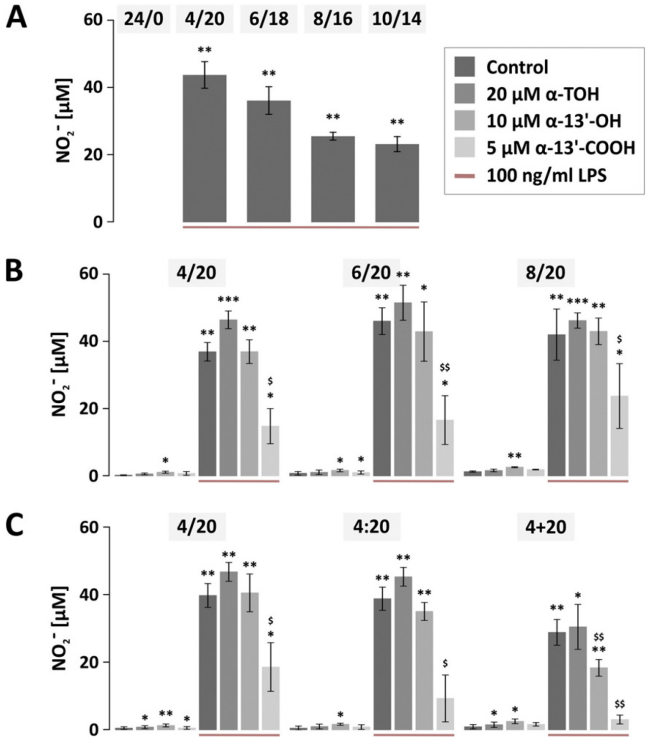

Fig. 2. Improved measurement of NO /nitrite in cell culture supernatants using the Griess assay. The incubation regimes described in Fig. 1 were applied to RAW264.7 macrophages using α-tocopherol (α-TOH) and its long-chain metabolites α-13′-hydroxychromanol (α-13′-OH) and α-13′-carboxychromanol (α-13′-COOH) as test compounds. (A) The combination of 4 h pre-incubation and 20 h LPS stimulation revealed the strongest and most reliable induction of NO in the LPS samples. (B) The shorter pre-incubation periods (4/20) resulted in a slightly pronounced anti-inflammatory effect of our test compound α-13′-COOH (4/20 vs. 8/20). (C) The size of the anti-inflammatory effect of the LCMs was strongest and most reliable when a complete change of the incubation medium after a 4 h pre-incubation period was applied. Mean levels of three independent biological experiments are shown. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, * vs. control; $ vs. LPS treatment.

setup was optimized in this study and varied in incubation time (24 to 28 h) and total incubation volume used (1 to 2 ml DMEM without serum per well). The experiments were performed as three independent biological experiments, each with one well per sample for incubation (24-well plate) that was split to four wells per sample for technical replicate measurements (96-well plate). More details are provided in the results section.

2.2. Griess assay

The Griess assay itself was optimized by reducing the amount of Griess reagent (normally 1:1 dilution), while the overall performance of the assay was maintained. 150 µl incubation supernatant (centrifuged: 400 ×*g*, 5 min) were mixed with 130 µl de-ionized water and 20 µl Griess reagent (#ALX-400-004-L050, Enzo Life Sciences, Germany).

3. Results

The test compounds used for optimizations described here are the long-chain metabolites (LCM) of vitamin E, in particular the LCM of α-tocopherol (TOH), i.e. α-13′-hydroxychromanol (α-13′-OH) and α-13′-carboxychromanol (α-13′-COOH). More details about the LCM in general or about their biological effects are on hand for further reading (Schmölz et al., 2016). When LPS is used as inflammatory stimulus, mainly high concentrations of LPS (1 mg/ml) are used in literature (Jin et al., 2016). However, we used a milder stimulus of 100 ng/ml LPS (#L2630, Sigma-Aldrich, Germany; stock: 1 mg/ml in de-ionized water) to avoid saturation effects.

The incubation regime is critical to the outcome of the experiment, in our hands and also in Robbins' (Robbins et al., 2016). However, many different regimes are used for the incubation of cells: pre-incubation with LPS (Robbins et al., 2016), co-stimulation (Jin et al., 2016; Robbins et al., 2016) or pre-treatment with the test compound (Li et al., 2017; Robbins et al., 2016) have been described. Also the total incubation time varies from 18 h (Robbins et al., 2016) to 48 h (Wallert et al., 2015), with 24 h being most popular (BenSaad et al., 2017; Li et al., 2017).

We started our optimizations with a total incubation time of 24 h, divided into a pre-incubation period (test substances only, 1 ml DMEM without serum per well) and a co-incubation period (simple addition of LPS). The pre-incubation periods varied from 4 h to 10 h, with an appropriate co-incubation period of 20 h to 14 h (Figs. 1 and 2A). The LPS induced release of NO was sufficiently above 20 µM NO, which met our criteria outlined before. Time-dependent effects were obvious: LPS induced release of NO was diminished by shorter co-incubation periods (20 h incubation with LPS: 44.1 ± 4.0 µM; 14 h incubation with LPS: 23.4 ± 2.2 µM, $p < 0.01$).

Next, the effect of variations in pre-incubation periods (from 4 h to 8 h) combined with a co-incubation time with LPS of 20 h was tested (Figs. 1 and 2B). Obviously, the shorter pre-incubation periods resulted in a slightly pronounced anti-inflammatory effect of our test compound α-13′-COOH (4 h pre-incubation: 14.9 ± 5.2 µM vs. 8 h pre-incubation: 23.8 ± 9.6 µM). In our hands (not shown), the effect of 4 h pre-incubation followed by 20 h co-incubation was slightly more reliable than the combination of 6 h pre-incubation followed by 20 h co-incubation. We therefore decided to continue with a pre-incubation time of 4 h.

Another modification of the incubation regime enabled the reliable evaluation of small anti-inflammatory effects. For this, the pre- and co-incubation times were fixed at 4 h and 20 h, respectively, but the application mode for LPS and test compound was changed. Beside (i) simple addition of LPS (no replacement of incubation medium, 1000 µl in total for 24 h), (ii) partly exchange (500 µl of 1000 µl replaced after 4 h, 500 µl old incubation medium and 500 µl new incubation medium for 20 h, 1500 µl in total), or (iii) total replacement of the incubation medium was studied (1000 µl incubation medium for 4 h, medium removed, 1000 µl new incubation medium for 20 h, 2000 µl in total; Figs. 1 and 2C). With increasing total amounts of the test compound, a diminished effect of the LCM α-13′-COOH on the LPS-stimulated release of NO was found [(i) 18.6 ± 7.2 µM, (ii) 9.4 ± 6.9 µM, (iii) 3.1 ± 1.3 µM; (i) vs. (iii) $p < 0.05$]. The total replacement of the incubation medium also resulted in prominent inhibition for α-13′-OH [(i) 40.6 ± 5.6 µM, (ii) 35.1 ± 2.7 µM, (iii) 18.4 ± 2.5 µM; (i) and (ii) vs. (iii) $p < 0.05$)], which was not observed before.

4. Conclusion

In our hands, this procedure provides most reliable results and a cost-effective sample preparation. Our optimization steps emphasize the need of critically evaluating the experimental setup of the Griess assay. We recommend to using our protocol as a starting point for own adoptions to particular needs.

Competing interests

The authors declare no competing interests.

Acknowledgements

The work of SL is supported by grants from the Federal Ministry of Education and Research (01EA1411A), the Deutsche Forschungsgemeinschaft (DFG; RTG 1715 and CRC/TR 124) and the German Ministry of Economics and Technology (AiF 16642 BR) via AiF (German Federation of Industrial Research Associations) and FEI (Research Association of the German Food Industry) as well as by the Free State of Thuringia and the European Social Fund (InflammAging 2016 FGR 0045). The work of LS is supported by the Free State of Thuringia and the European Social Fund (InflammAging 2016 FGR 0045). The work of MW is funded by the DFG (Wa 3836/1-1).

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6.3 Manuskript 3

1524 DOI 10.1002/mnfr.201400737 *Mol. Nutr. Food Res.* 2015, *59*, 1524–1534

RESEARCH ARTICLE

α-Tocopherol long-chain metabolite α-13′-COOH affects the inflammatory response of lipopolysaccharide-activated murine RAW264.7 macrophages

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Received: October 13, 2014
Revised: April 13, 2015
Accepted: April 13, 2015

Scope: Inflammatory response of macrophages is regulated by vitamin E forms. The long-chain metabolite α-13′-carboxychromanol (α-13′-COOH) is formed by hepatic α-tocopherol (α-TOH) catabolism and acts as a regulatory metabolite via pathways that are different from its metabolic precursor.

Methods and results: Using semisynthetically-derived α-13′-COOH we profiled its action on LPS-induced expression of pro- and anti-inflammatory genes using RT-qPCR and of key proteins by Western blotting. Effects on inflammatory response were assessed by measuring production of nitric oxide and prostaglandin (PG) E_2, PGD_2, and $PGF_{2\alpha}$. α-13′-COOH inhibits proinflammatory pathways in LPS-stimulated RAW264.7 macrophages more efficiently than α-TOH. Profiling inflammation-related genes showed significant blocking of interleukin (Il)1β by the metabolite and its precursor as well, while upregulation of Il6 was not impaired. However, induction of Il10, cyclooxygenase 2 (Cox2) and inducible nitric oxide synthase (iNos) by LPS and consequently the formation of nitric oxide and PG was significantly reduced by α-13′-COOH. Interestingly, α-13′-COOH acted independently from translocation of NFκB subunit p65.

Conclusion: Our study sheds new light on the mode of action of α-TOH on the inflammatory response in macrophages, which may be mediated in vivo at least in part by its metabolite α-13′-COOH. Our data show that α-13′-COOH is a potent anti-inflammatory molecule.

Keywords:
α-Tocopherol /α-13′-COOH /Inflammation /Inflammatory response /Macrophages / Macrophage activation

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Abbreviations: **α-CEHC**, α-(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H)-1-benzopyran-2-propanoic acid; **α-13′-COOH**, α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid; **α-LCM**, α-tocopherol long-chain metabolite; **α-TOH**, α-tocopherol; **COX**, cyclooxygenase; **iNOS**, inducible nitric oxide synthases; **NFκB**, nuclear factor kappa-light-chain-enhancer of activated B cell; **PG**, prostaglandins; **PKC**, protein kinase C; **TNF**, tumor necrosis factor

1 Introduction

The inflammatory response of macrophages is a key initiator and driving force for many age-related diseases such as atherosclerosis [1]. Activated macrophages excessively accumulate lipids that lead to foam cell formation and the release of signaling molecules, such as chemokines, proinflammatory cytokines (tumor necrosis factor (TNF) α, IL-1β and IL-6, nitric oxide, and prostaglandins (PG) [2]. The inflammatory response is mainly triggered by the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Activation of NFκB and the translocation of its p65 subunit to the nucleus is caused by extracellular stimuli, such as LPS, resulting in ubiquitination and phosphorylation of the regulatory protein subunit IκB by IκB kinase [3]. Subsequently, inflammatory enzymes, such as cyclooxygenases (COX) and inducible nitric oxide synthases (iNOS), are activated. Whereas COX1 is constitutively expressed, COX2 is induced under acute and chronic inflammatory conditions and generates proinflammatory eicosanoids, including PGE_2, PGD_2, and $PGF_{2\alpha}$. Nitric oxide synthases comprise a family of three isoforms of which iNOS catalyzes the formation of nitric oxide from L-arginine in μM amounts.

Vitamin E forms differ by methylation patterns of the hydroxychromanol ring and saturation of the side-chain (α-, β-, γ-, δ-tocopherol, and -tocotrienol) [4]. Within the group of vitamin E, α-tocopherol (α-TOH) is assigned as the most biologically important vitamer known for its anti-oxidative and nonanti-oxidative properties. α-Tocopherol regulates the expression of genes, e.g. of proteins involved in uptake, transport, degradation, and excretion of tocopherols, such as α-tocopherol transfer protein [5], cytochrome P450 (CYP) 3A4 [6], and/or CYP4F2 [7], multidrug resistance 1 [8], lipoprotein uptake, such as cluster of differentiation 36 [9], inflammation, and regulation of signal transduction, e.g. via peroxisome proliferator-activated receptor γ [10].

In the liver, α-TOH is metabolized by side-chain truncation initiated by CYP4F2/CYP3A4-dependent ω-hydroxylation [11]. Subsequent α-oxidation in peroxisomes forms the carboxychromanol α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid (α-13′-COOH) that occurs in human serum as recently shown by our group [9]. Further β-oxidation cycles in peroxisomes and mitochondria result in the formation of α-carboxyethyl-hydroxychroman (α-CEHC, α -(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H)-1-benzopyran-2-propanoic acid), the main catabolic end-product of α-TOH metabolism [12, 13].

The inhibition of the inflammatory response by tocopherol and tocotrienol forms has been investigated, but the effects of α-tocopherol long-chain metabolite (α-LCM) remain poorly understood. Jiang et al. reported that 9′-COOH and 13′-COOH inhibit COX activity in epithelial A549 cells [14]. We therefore investigated the modulation of the inflammatory response of murine RAW264.7 macrophages by the physiological α-LCM α-13′-COOH. The reason for investigating α-13′-COOH over other vitamin E metabolites is its proposed physiological relevance as indicated by its presence in human serum and our expertise to synthesize this α-LCM, which is commercially not available, from the natural product garcinoic acid. Further, we analyzed the underlying signal transduction processes. Our results reported here are in good agreement with recently published data by our group that characterize α-13′-COOH as a member of a new class of signaling molecules that show higher bioactivity than α-TOH [9]. We therefore assume a new molecular mode of action of α-TOH via its LCM α-13′-COOH in regulating the inflammatory response of macrophages.

2 Materials and methods

2.1 Chemicals

If not indicated otherwise chemicals were obtained from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), or Merck Millipore (Darmstadt, Germany).

2.2 RAW264.7 macrophage culture

Murine RAW264.7 macrophages (ATCC, Manassas, VA) were cultivated in 150 cm^2 cell culture flasks in high glucose (4.5 g/L) DMEM supplemented with 10% FBS and 0.1 mg/mL penicillin/streptomycin/L-glutamine mixture. For further culturing, cells were scraped from the cell culture flask and split in a mixture of 70% fresh high glucose DMEM and 30% used culture DMEM obtained from previous periods of RAW264.7 macrophage culture as recommended by ATCC. Cells were split thrice a week at a confluence of about 80% and cultured at 37°C in humidified 5% CO_2/95% air atmosphere. For experiments cells were seeded, cultured for 24 h and then incubated with compounds as indicated in the figures. Cells were harvested for further processing as described below.

2.3 RNA isolation and cDNA synthesis

Total RNA was isolated from cell lysates using Qiagen RNeasy Mini kit (Hilden, Germany) including on-column DNase I digestion (Qiagen) as described earlier [15]. cDNA synthesis was performed using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), 5 μg of total RNA and 500 ng/μL oligo-dT primers as outlined previously [16].

2.4 Quantitative real-time RT-PCR (RT-qPCR)

Quantitative real-time RT-PCR was performed on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using QuantiTect SYBR® Green PCR kit (Qiagen)

as reported earlier [16]. Primers (Il6, Il1β, Il10, Tnfα, iNos, Cox2, and Ppib; see Table 1) were purchased from Invitrogen (Karlsruhe, Germany). The PCR runs included a 15 min preincubation at 95°C, followed by a 40 cycle two-step PCR consisting of a denaturing phase at 94°C for 15 s and a combined annealing and extension phase at 60°C for 30 s. Results were analyzed using LightCycler software 1.5.0.39.

2.5 Immunoblotting

2.5.1 iNos and Cox2

Cells were harvested using a nondenaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 15 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) containing 1% protease inhibitor (Fisher Scientific, Schwerte) and mixed 3:1 with loading buffer (6.26% 1 M Tris-HCl, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue). Cellular proteins were separated by SDS-PAGE and transferred to PVDF membranes (VWR, Darmstadt, Germany) using a transfer buffer containing 0.25 M Tris, 1.92 M glycine, 0.1% SDS and 20% methanol (pH 8.3). For iNos and Cox2 antibodies were incubated in SignalBoost™ Immunreaction Enhancer kit (Calbiochem, Darmstadt, Germany), and for α-tubulin a hybridization buffer containing 0.5% milk powder and PBS (0.137 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 x 2 H_2O, 1.5 mM KH_2PO_4, pH 7.4) was used, respectively. Primary antibodies mouse anti-iNos (clone 6), rabbit anti-Cox2 (clone EP1978Y), and mouse anti-α-tubulin (clone B-5-1-2) were from BD Biosciences (Heidelberg, Germany), Epitomics (Burlingame, CA), and Sigma-Aldrich, respectively. Secondary antibodies (rabbit anti-mouse and swine anti-rabbit both labeled with horseradish peroxidase) were from DAKO (Hamburg, Germany).

2.5.2 NFκB

Cells were scraped in warm PBS, sedimented and resuspended in nondenaturing buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.025% NP-40) containing 2% protease inhibitor. For subcellular fractionation, cells were vortexed and incubated on ice. Aliquots of the total fraction were taken and samples were then centrifuged (3600 × *g*, 10 min, 4°C). The supernatant containing the cytosolic fraction and the pellet with the nuclear fraction were separated. Both fractions were resuspended in nondenaturing buffer (50 mM Tris-HCl, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 1 mM EDTA). All fractions were sonicated three times for 5 s and mixed with loading buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membranes as outlined above. Primary antibodies against NFκB subunit p65 (rabbit anti-p65, clone E379, 1:1000) from Epitomics, mouse anti-α-tubulin (clone B-5-1-2, 1:5000) and rabbit anti-PARP (clone 46D11, 1:1000) from Cell Signaling Technology (Danvers, MA) were used. Secondary antibodies (rabbit anti-mouse or swine anti-rabbit labeled with horseradish peroxidase, 1:5000) were from DAKO. For p65 and PARP the SignalBoost™ Immunreaction Enhancer Kit was used.

2.5.3 Detection

For detection Pierce ECL Western Blotting Substrate and CL-XPosure™ Films (Thermo Scientific, Rockford, IL) were applied. Blots were analyzed densitometrically using ImageJ software version 1.4.3.67.

2.6 Quantification of nitric oxide formation using Griess assay

RAW264.7 macrophages were seeded in a mixture of 70% fresh supplemented high glucose DMEM and 30% used culture medium for 24 h. For experiments, adherent cells were washed twice and incubated with either 20 μM α-TOH or 5 μM α-13′-COOH in fresh medium free high glucose DMEM for further 24 h followed by another 24 h incubation of both 100 ng/ml LPS and either solvent (DMSO), 20 μM α-TOH, or 5 μM α-13′-COOH. For Griess assays, the cell culture supernatants were removed and centrifuged (400 × *g*, 5 min, room temperature) to remove cells. The supernatants were diluted with water (1:0.87), mixed with Griess reagent (15%) and incubated in the dark. The concentration of nitrite in the supernatants was measured at 544 nm using a BMG Labtech FLUOstar omega and MARS data analysis software version 2.41.

2.7 Prostaglandin E_2 quantification via ELISA

RAW264.7 macrophages were seeded in supplemented high glucose DMEM as described above for 24 h and incubated with either solvent, 5 μM α-13′-COOH or 100 μM α-TOH for 24 h followed by another 24 h of coincubation with 100 ng/mL LPS and either 5 μM α-13′-COOH or 100 μM α-TOH. Next, cell culture supernatants were collected and immediately stored on ice until centrifugation (2000 × *g*, 10 min, 4°C) for removing cells and debris. Release of PGE_2 by macrophages was quantified using the Prostaglandin E_2 Enzyme Immunoassay Kit (Biotrend, Cologne) as indicated in the manufacturer's protocol. In brief, 100 μL of each sample or standard were mixed with 100 μL assay buffer. The samples were then pipetted in microtiter plate wells coated with goat anti-mouse IgG. Twenty-five microliters PGE_2 peroxidase conjugates and 25 μL monoclonal mouse PGE_2 antibody solution were added. If necessary, supernatants were diluted to comply with the linear measurement range of the assay. The sealed plate was shaken for 2 h at room temperature. After incubation, the plate was washed four times with

washing buffer, dried by tapping out the liquid and incubated with 100 μL tetramethylbenzidine substrate per well for 30 min. The reaction was stopped by adding 50 μL Stop Solution (Biotrend) per well. The concentration of PGE_2 in the supernatants was measured by 450 nm in a Multiscan™ GO Microplate Spectrophotometer (Thermo Scientific). To exclude unspecific binding signals, absorption of blanks was subtracted from that of samples. The amount of PGE_2 in the supernatants was calculated using a standard curve.

2.8 Prostaglandin quantification using RP LC and MS

Eicosanoids were extracted and separated on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters, Milford, MA) using an Acquity™ UPLC system (Waters, Milford, MA, USA) as previously described [17]. In brief, chromatography was performed at a flow rate of 0.8 mL/min and a column temperature of 45°C. The solvents for the mobile phase were (A) acetonitrile and (B) water/acetonitrile (90/10) both acidified with 0.07% v/v formic acid. Isocratic elution at A/B = 30/70 was performed for 2 min and followed by a linear gradient to A/B = 70/30 within 5 min. The chromatography system was coupled to a QTRAP 5500 Mass Spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray ionization source. Eicosanoids were analyzed by multiple reaction monitoring. The parameters were adjusted as described [18]. Automatic peak integration was performed with Analyst software version 1.6 (AB Sciex, Darmstadt, Germany) using IntelliQuan default settings. Data were normalized on the internal standard PGB_1 and are given as relative intensities. The reported method was optimized to compare eicosanoid profiles between samples and not for absolute quantification.

2.9 Semisynthesis of α-LCMs

All chemicals were used as received from the supplier. Isolation of the educt garcinoic acid from the African bitter nut *Garcinia kola* and synthesis of the α-LCM were performed as previously described [19, 20].

2.10 Statistics

Data are presented either as means ± SD or as means ± SEM of independent experiments as indicated. In order to test for statistical significance paired Student's *t*-tests were performed using Microsoft Excel 2007/2010. For calculating the amounts of PG, Anova, and Tukey post-hoc tests with logarithmized values were used.

3 Results

It has been quite well studied that the inhibitory capacity of tocopherols and tocotrienols as well as their modes of action on proinflammatory pathways depends on the vitamer [21, 22]. For example, cyclooxygenase activity is inhibited in vitro by α- and β-TOH, γ-TOH, γ-tocotrienol, and δ-TOH with decreasing effectiveness. Beside the vitamers, the carboxychromanol metabolites δ- and γ-9′-COOH as well as δ- and γ-13′-COOH are more potent anti-inflammatory agents compared to their metabolic precursors δ-TOH and γ-TOH in inhibiting COX1 and COX2 activity [14]. We therefore analyzed the anti-inflammatory action of α-13′-COOH, a physiological α-LCM recently identified in human serum by our group [9]. Studies in THP-1 macrophages defined an EC_{50} value of 7.4 μM ± 1.5 μM for α-13′-COOH but no cytotoxicity was observed for α-TOH up to 100 μM using sulforhodamine B assays [9]. Therefore, we used 5 μM of α-13′-COOH that was obtained semisynthetically [20] and a maximum of 100 μM of α-TOH as reference for the functional studies on mouse RAW264.7 macrophages outlined here. If not indicated otherwise, cells were preincubated with α-13′-COOH or α-TOH for 24 h prior to coincubation of the compounds with 100 ng/mL LPS for another 24 h. We investigated α-13′-COOH in comparison to α-TOH in all experiments in order to get first insights in the importance of the side-chain oxidation for the biological function and effectiveness of the tocopherol metabolite.

3.1 Lipopolysaccharide-induced upregulation of Il1β, Il10, and Tnfα mRNAs but not of Il6 is blocked by α-13′-COOH

Previous studies have shown that α-TOH inhibits the inflammatory response of macrophages [3, 23–25]. We therefore investigated the effect of α-13′-COOH on the LPS-induced inflammatory response of mouse RAW264.7 macrophages. To analyze the effect of α-13′-COOH on the expression of typical LPS-responsive genes, we first focused on Il1β and Tnfα as pro-inflammatory mediators, on Il6 as an ambivalent inflammatory mediator and Il10 as an anti-inflammatory one. As shown in Fig. 1, neither 5 μM α-13′-COOH nor 100 μM α-TOH had any effects on the basal expression levels of the four selected marker genes. As expected, LPS induced significantly the expression of Il1β (2000-fold, $p < 0.001$), Il6 (12 000- to 16 500-fold, $p < 0.001$), and Tnfα (15- to 34-fold, $p < 0.001$) as well as Il10 (15- to 23-fold, $p < 0.05$). Treatment with neither 5 μM α-13′-COOH nor with 100 μM α-TOH affected the LPS-induced expression of Il6 (Fig. 1A). α-13′-COOH but not α-TOH tended to decrease LPS-induced expression of Tnfα (Fig. 1B). In contrast, LPS-induced upregulation of Il1β was significantly reduced by both α-13′-COOH and α-TOH by 94 and 61%, respectively ($p < 0.05$, $p < 0.01$; Fig. 1C). Further, α-13′-COOH blocked LPS-induced upregulation of Il10 mRNA by 89% ($p < 0.05$), whereas α-TOH

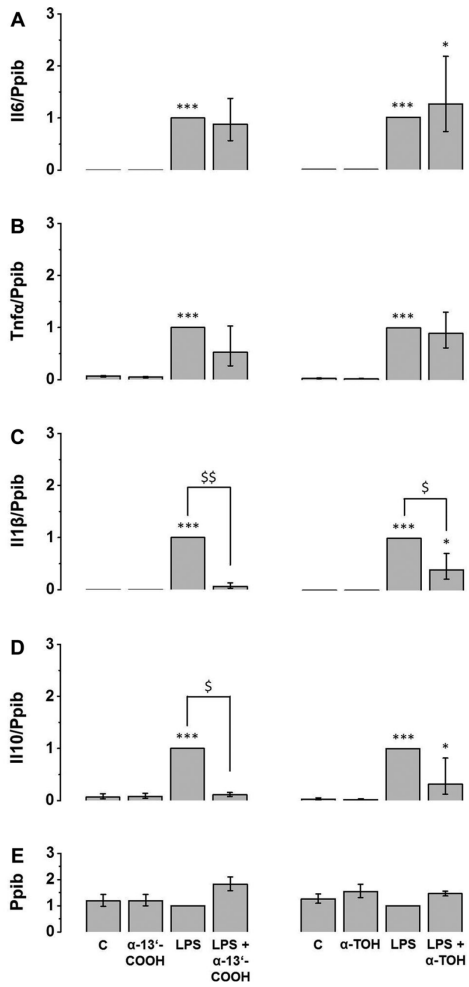

Figure 1. α-13′-COOH differently affects the expression of LPS response genes in mouse RAW264.7 macrophages. Murine RAW264.7 macrophages were preincubated with either solvent, 5 μM α-13′-COOH (left) or 100 μM α-TOH (right) for 24 h followed by coincubation of 100 ng/mL LPS with either solvent (DMSO), α-13′-COOH, or α-TOH for another 24 h. Appropriate control macrophages were cultured in the presence of either solvent or compound only for the same time periods; samples cultured for 24 h in the presence of solvent followed by another 24 h with LPS served as reference and were defined as 1. Expression levels of the inflammatory response genes Il6, Tnfα, Il1β, and Il10 were measured using RT-qPCR and normalized to the expression of the reference gene Ppib. (A) The α-LCM α-13′-COOH and its precursor α-TOH did not block LPS-induced upregulation of Il6 mRNA levels. (B) LPS-induced expression of Tnfα mRNA tended to be decreased by α-13′-COOH by 48%, whereas α-TOH showed no effect. (C) LPS-upregulated expression of Il1β mRNA was significantly blocked by α-13′-COOH and α-TOH by 94% ($p < 0.01$) and 61% ($p < 0.05$), respectively. (D) The induction of Il10 expression by LPS was significantly reduced by α-13′-COOH by 89% ($p < 0.05$), and by 81% by α-TOH. (E) Expression of the reference gene Ppib remained unchanged under all conditions. Error bars display calculated maximum and minimum expression levels representing SEM expression levels of four to five independent biological experiments, each measured in one technical replicate. $^{*}p < 0.05$; $^{***}p < 0.001$ (versus solvent control); $^{\$}p < 0.05$; $^{\$\$}p < 0.01$ (versus LPS treatment).

had no significant effect (Fig. 1D). The reference gene Ppib remained unchanged under all conditions.

3.2 Lipopolysaccharide-induced upregulation of Cox2 and iNos is blocked by α-13′-COOH but not by α-TOH

Expression of Cox2 and iNos is also induced by LPS, and α-TOH has been considered to modulate this upregulation [3, 23]. We therefore wanted to know whether the metabolite of α-TOH, α-13′-COOH, modulates the response of Cox2 and iNos to LPS in RAW264.7 macrophages. Neither α-13′-COOH nor α-TOH influenced the basal expression of Cox2 and iNos at the mRNA and protein level in our model (Fig. 2). LPS significantly increased the expression of both Cox2 mRNA (2600- to 3700-fold, $p < 0.001$; Fig. 2A) and iNos mRNA (210- to 300-fold, $p < 0.001$; Fig. 2B). α-13′-COOH inhibited the LPS-induced upregulation of Cox2 mRNA by 84% ($p < 0.01$) and iNos mRNA by 92% ($p < 0.001$); thus, α-13′-COOH was even more effective at lower concentrations than α-TOH that tended to block the LPS-induced upregulation of Cox2 mRNA by 59% (Fig. 2A) and iNos mRNA by 37% (Fig. 2B) at a concentration of 100 μM, respectively. Preincubation of α-13′-COOH for 24 h followed by another 24 h of coincubation of α-13′-COOH and 100 ng/mL LPS did not reveal any regulatory effects of α-13′-COOH on LPS-induced Cox2 protein levels (Fig. 2C). However, LPS-upregulated Cox2 protein levels were blocked significantly after 14 h of coincubation of LPS and α-13′-COOH by 75% ± 24% ($p < 0.001$; Fig. 2E), while upregulation of iNos protein by LPS was significantly inhibited by α-13′-COOH after 24 h by 74% ± 2% ($p < 0.001$; Fig. 2D). Neither upregulation of Cox2 protein nor iNos protein by LPS was affected by α-TOH.

3.4 Formation of PGE_2, PGD_2, $PGF_{2\alpha}$, and nitric oxide in RAW264.7 macrophages is blocked by α-13′-COOH

Cyclooxygenase 2 and iNos synthesize the signaling molecules PG and nitric oxide, which play a key role in the LPS-induced immune response of macrophages. As shown

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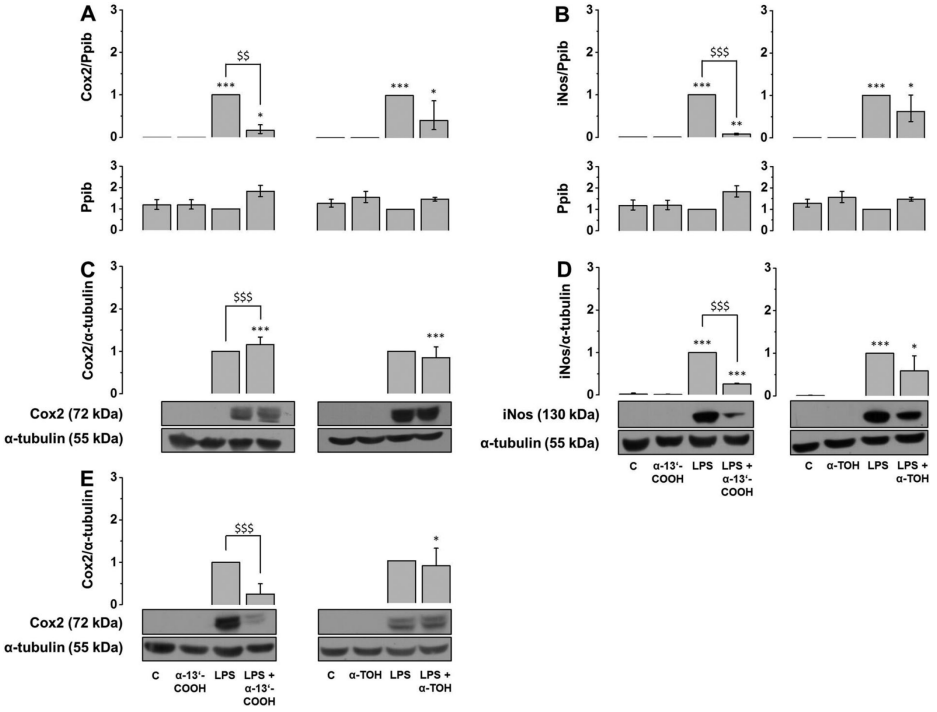

Figure 2. Lipopolysaccharide-induced expression of Cox2 and iNos is inhibited by α-13′-COOH in mouse RAW264.7 macrophages. RAW264.7 macrophages were incubated as described in the legend of Fig. 1 to perform RT-qPCR analyses (A, B) or for Western blot analyses (C, D). Additionally, Cox2 protein expression was analyzed after 14 h of coincubation (E). Control samples were defined as 1 (for details, see Fig. 1). Messenger RNA levels were normalized to Ppib mRNA and protein levels were normalized to α-tubulin for histogram quantification of Western blots. Lipopolysaccharide-induced upregulation of (A) Cox2 mRNA and (B) iNos mRNA was significantly reduced by α-13′-COOH by 84% (SEM min 7%, SEM max 14%, $p < 0.01$) and 92% (SEM min 1%, SEM max 2%, $p < 0.001$), respectively; α-TOH tended to reduce the upregulation of Cox2 and iNos by LPS by 59% (SEM min 22%, SEM max 46%) and 37% (SEM min 24%, SEM max 39%), respectively. Expression of Ppib remained unchanged under all conditions. (C) Lipopolysaccharide-induced Cox2 protein levels were not reduced by α-13′-COOH after 24 h. (E) Using earlier time points, 14 h, LPS-induced Cox2 protein levels were significantly reduced by α-13′-COOH by 75% ± 24% ($p < 0.001$). (D) Stimulated iNos protein levels were significantly reduced by α-13′-COOH by 74% ± 2% ($p < 0.001$) compared to LPS-stimulated cells. α-TOH failed to block the LPS-stimulated expression of Cox2 and iNos. Expression of α-tubulin remained unchanged under all conditions. The Western blots are representative examples of the blots used for densitometry. (A, B) Error bars display calculated maximum and minimum expression levels representing SEM expression levels of four to five independent biological experiments, each measured in one technical replicate. (C–E) Means of three independent biological experiments measured in one (iNos) or two (Cox2) technical replicates are shown; error bars represent SDs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (versus control); $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ (versus LPS treatment).

in Fig. 2, expression of Cox2 and iNos is inhibited at the mRNA and protein levels by the α-LCM α-13′-COOH in LPS-stimulated macrophages. Hence, we investigated the effect of α-13′-COOH on the LPS-stimulated production of PG and nitric oxide by Cox2 and iNos, respectively, in RAW264.7 macrophages. Treatment with 100 ng/mL LPS significantly induced the release of PGE_2 by RAW264.7 up to 18.3 ng/mL ± 4.1 ng/mL ($p < 0.05$) as measured by ELISA. α-Tocopherol decreased the LPS-induced release of PGE_2 to 7.9 ng/mL ± 2.4 ng/mL ($p < 0.05$), and the α-LCM blocked the LPS-mediated production of PGE_2 almost to baseline (1.5 ng/mL ± 1.2 ng/mL; $p < 0.05$) as shown in Fig. 3A. In addition, we determined PGE_2, PGD_2, and $PGF_{2\alpha}$ levels using UPLC-MS/MS (Fig. 3B–D). As expected, basal release of these Cox2-derived PG remained almost unaffected by both α-13′-COOH α-TOH, whereas treatment with 100 ng/mL LPS significantly induced their release. α-Tocopherol did not or hardly decrease PGE_2 (0.8 ± 0.4), $PGD_{2\alpha}$ (0.9 ± 0.5), and $PGF_{2\alpha}$ (0.7 ± 0.2),

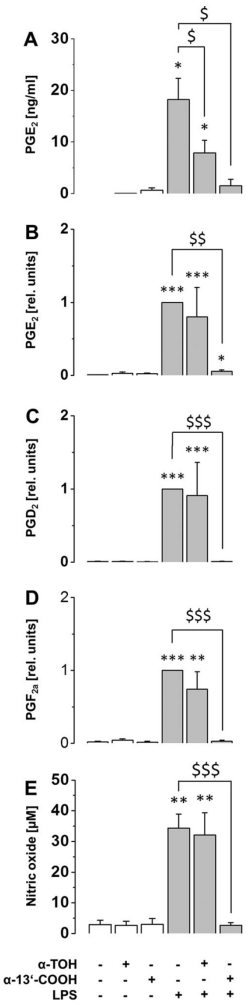

Figure 3. Release of PG and nitric oxide in LPS-stimulated mouse RAW264.7 macrophages is blocked by α-13′-COOH. RAW264.7 macrophages were incubated as described in Fig. 1 (gray bars), except the adapted α-TOH concentration used for Griess assay (20 μM); appropriate control cells are shown as white bars. Release of PG into the cell culture supernatants was measured by (A) ELISA and (B–D) UPLC-MS/MS. (A–D) Basal release of PG remained almost unaffected by both α-13′-COOH or α-TOH. Treatment with 100 ng/mL LPS significantly induced PG levels in the supernatant of RAW264.7 macrophages. (A) PGE_2 concentration increased up to 18.3 ± 4.1 ng/mL ($p < 0.05$) under LPS treatment. Coincubation with α-TOH and α-13′-COOH decreased PGE_2 concentration to 7.9 ± 2.4 ng/mL ($p < 0.05$) and nearly to baseline levels of 1.5 ± 1.2 ng/mL ($p < 0.05$), respectively. (B–D) In UPLC-MS/MS measurements revealed that α-TOH tends to decrease PGE_2, PGD_2, and $PGF_{2\alpha}$ concentrations, whereas α-13′-COOH significantly decreased PG nearly to baseline levels ($p < 0.001$). (E) Culture supernatants were used for quantifying the release of nitric oxide using Griess assays. Basal nitric oxide levels were affected neither by α-13′-COOH nor α-TOH. Treatment with LPS increased the formation of nitric oxide significantly up to 34.3 μM ± 4.5 μM ($p < 0.01$). The LPS-induced increase of nitric oxide release was significantly blocked by α-13′-COOH down to almost baseline levels (2.7 μM ± 0.9 μM, $p < 0.001$), but not by α-TOH (32.1 μM ± 7.4 μM). Means of (A) three or (E) four independent biological experiments performed each in (A–D) one or (E) four technical replicates are shown; error bars display standard errors. $^{*}p < 0.05$, $^{**}p < 0.01$ (versus control); $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ (versus LPS treatment).

whereas α-13′-COOH significantly blocked the LPS-induced release of the PG almost to baseline levels (PGE_2: 0.06 ± 0.02, PGD_2: 0.01 ± 0.00 and $PGF_{2\alpha}$: 0.03 ± 0.01; $p < 0.001$). We also measured the effects of α-13′-COOH and α-TOH on the formation of nitric oxide in RAW264.7 macrophages activated by LPS. Treatment with LPS increased the concentration of nitric oxide in the culture supernatant to 34.3 μM ± 4.5 μM ($p < 0.01$). In our experimental setup, we observed no significant effect of α-TOH on the LPS-triggered induction of nitric oxide production. In contrast, α-13′-COOH significantly decreased the LPS-induced formation of nitric oxide down to 2.7 ± 0.9 μM ($p < 0.001$; Fig. 3B). Neither α-TOH nor α-13′-COOH influenced the basal release of nitric oxide in nonstimulated RAW264.7 macrophages.

3.5 Lipopolysaccharide-induced translocation of NFκB is not affected by α-13′-COOH

We were next interested whether the immunomodulatory properties of α-13′-COOH are mediated via the key regulator of the LPS response in macrophages, namely NFκB. As shown in Fig. 4 treatment of 1 μg/mL LPS for one hour resulted in a significant translocation of the NFκB subunit p65 from the cytosol to the nucleus while the amount of total and cytosolic p65 remained unaffected. Neither α-13′-COOH nor α-TOH caused significant changes in the total, cytosolic or nuclear amount of p65 in the absence of LPS. However, α-13′-COOH failed to block the translocation of the p65 subunit into the nucleus (Fig. 4A, left), whereas α-TOH significantly enhanced translocation of p65 by 54 ± 20% ($p < 0.05$; Fig. 4A, right). The total cell and cytosolic fractions did not show any changes in the amount of the p65 subunit during coincubation with LPS and either α-13′-COOH or α-TOH (Fig. 4B and C). The reference proteins α-tubulin (for the cytosolic fraction and total cell lysate) and PARP (for the nuclear fraction) remained unchanged under all conditions.

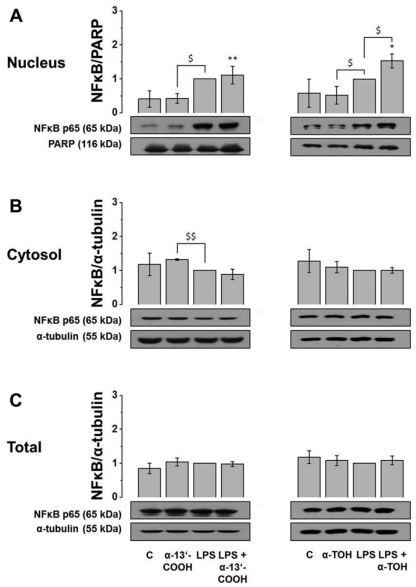

Figure 4. Inhibitory effects by α-13′-COOH are independent from the translocation of the NFκB subunit p65. Murine RAW264.7 macrophages were preincubated with solvent, 2.5 μM α-13′-COOH or 100 μM α-TOH for 24 h followed by 1 μg/mL LPS for one further hour. Separated nuclear (A) or cytosolic (B) fractions and total cell lysates (C) were used to analyze translocation of the NFκB p65 subunit. PARP (A) and α-tubulin (B, C) were used as reference proteins that remained unchanged under all conditions. (A) Lipopolysaccharide-induced translocation of the p65 subunit from the cytosol into the nucleus that was not blocked by α-13′-COOH (left). Interestingly, p65 translocation was augmented significantly by α-TOH to 154% ± 20% ($p < 0.05$) compared to LPS-stimulated samples (right). (B) In the cytosol no changes in p65 subunit levels were observed during coincubation with LPS and α-13′-COOH or α-TOH compared to LPS-stimulated samples. In the cytosolic fraction levels of the p65 subunit were slightly but significantly higher after α-13′-COOH incubation by 32% ± 2% ($p < 0.01$) versus stimulation with LPS, whereas α-TOH did not change p65 levels in the cytosolic fraction. Representative images of Western blots are shown. Means and standard deviations of three (α-13′-COOH) or four (α-TOH) independent biological experiments are shown. *$p < 0.05$, **$p < 0.01$; (versus control); $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ (versus LPS treatment).

4 Discussion and conclusions

α-Tocopherol is widely used to prevent oxidation of lipids and lipid compounds and inflammation-related diseases, although only a high-dose *RRR*-α-TOH supplementation suppresses oxidative stress status successfully [26]. Next to its antioxidant properties, vitamin E directly regulates signal transduction and expression of genes involved in inflammatory processes to different extents depending on the vitamer [27].

The release of chemokines, cytokines and mediators such as nitric oxide and PG by macrophages characterizes the inflammatory response. Vitamin E supplementation is known to decrease cytokine production in vivo [28], but the modes of action by which vitamin E affects the inflammatory response remain poorly understood. Studies analyzing the effect of α-TOH on LPS-induced upregulation of Cox2 expression differ in their results. Yam et al. found that all vitamin E forms except α-TOH impair LPS-induced gene expression of Cox2 in murine RAW264.7 macrophages [29], and Ng and coworkers showed that LPS- upregulation of Cox2 gene expression is inhibited by α-TOH in peritoneal macrophages [3]. On the other hand, upregulation of iNos by LPS is consistently diminished by α-TOH [3]. Next to TOHs, metabolites formed during hepatic catabolism of vitamin E, such as α- and γ-CEHC, are efficient inhibitors of the inflammatory response that act by blocking the expression of iNos and Cox2 in TNFα-stimulated EOC-20 microglial cells and LPS-stimulated murine RAW264.7 macrophages [29, 30].

In the present study, α-TOH has been used primarily as a reference. Therefore, we have used high concentrations that have been reported in studies so far [31, 32]. We used 5 μM of α-13′-COOH for all experiments, except for investigating the effects of the metabolite on the translocation of the NFκB subunit p65. To achieve activation of NFκB within one hour of incubation with LPS, higher concentrations of LPS of 1 μg/mL were required as shown by others [3, 33]. Since these high doses of LPS together with 5 μM of α-13′-COOH were highly stressful for RAW264.7 macrophages, we had to reduce the concentration of α-13′-COOH to 2.5 μM for these experiments in order to avoid that findings are due to cytotoxicity. As shown in Figs. 1 and 2, the metabolite, but not the precursor α-TOH, clearly regulate the expression after 24 h of preincubation with α-13′-COOH followed by co-incubation with α-13′-COOH and LPS. Unfortunately, results from Western blotting assays were inhomogeneous. Therefore we adapted the experimental conditions for Cox2 protein expression experiments by shortening the time of co-incubation with α-13′-COOH and LPS in line with work of others, who showed that LPS-induced expression of Cox2 is significantly induced already after 6 h [34].

We recently found the hepatic α-TOH metabolite α-13′-COOH in human serum of healthy nonsupplemented volunteers and provided first evidence that the α-LCM may represent a new class of regulatory signaling molecules [9]. We therefore wondered whether the α-LCM may affect the LPS-triggered inflammatory response of macrophages. Measurement of selected pro- and anti-inflammatory mediators showed that α-13′-COOH affects gene expression more effectively and differently than α-TOH (Figs. 1A–D, 2A and B). Whereas α-13′-COOH inhibited the LPS-induced expression of Il1β, the upregulated expression of Il6 and Tnfα is not significantly affected by α-13′-COOH. Since the expression of the proinflammatory gene Il1β is blocked by α-13′-COOH, also

the late response to inflammatory stimuli via the increased expression of Il10 does not occur in the presence of α-13′-COOH. These findings indicate that α-13′-COOH modifies the inflammatory response in LPS-stimulated macrophages, but these findings require further verification in vivo.

Cyclooxygenases and iNOS catalyze signaling molecule production and play therefore an important role in regulating inflammatory processes. Downregulation of iNos expression after α-TOH treatment has been observed by Ng and Ko in LPS-stimulated murine macrophages [3]. However, in our experiments α-TOH tended merely to block LPS-induced iNos mRNA and protein expression (Fig. 2B), possibly due to different incubation conditions and the cell line used in our study. Contradictory results for the effect of α-TOH on iNos expression or activity in vitro and in vivo have been reported also by others [35, 36], complicating the interpretation of our results. In agreement with others we confirmed that Cox2 expression is not affected by α-TOH [23, 29, 37]. In contrast to α-TOH, α-13′-COOH exhibits significant and reliable effects in LPS-stimulated macrophages on the expression of both iNos and Cox2 at the mRNA and protein levels (Fig. 2), which implicates a different mode of action of the α-LCM compared to α-TOH. Different incubation times have been used for iNos (24 h) and Cox2 (24 and 14 h) analyses at the protein level in our study as time course experiments revealed that activation of Cox2 occurs earlier than that of iNos (Fig. 2). This is possibly due to higher levels of expression of Cox2 compared to iNos or different modes or kinetics of the regulation.

In good agreement with the inhibition of iNos and Cox2 expression, the formation of PG and nitric oxide is also efficiently and reliably blocked by α-13′-COOH (Fig. 3); interestingly this takes place at much lower concentrations than for α-TOH. However, α-TOH significantly blocks PGE_2 as measured by ELISA (Fig. 3A) and in agreement with findings by Ng and Ko [3], but only tends to decrease PG production as found using UPLC-MS/MS analysis (Fig. 3B–D). It is therefore difficult to draw a clear conclusion for the effect of α-TOH on Cox2 product formation, but with respect to the high α-TOH concentrations used in our study, we consider our observation of minor importance. In agreement with iNos expression data, α-TOH did not block the LPS-induced production of nitric oxide in RAW264.7 macrophages (Fig. 3B). As already discussed, results on the anti-inflammatory effects of α-TOH are contradictory. Similar to our findings, Kim et al. reported no protection against the LPS-induced production of nitric oxide by α-TOH in RAW264.7 macrophages [37], whereas others found significant blocking of the LPS-induced production of nitric oxide by α-TOH [3, 29]. For the analyses of PG and nitric oxide, different α-TOH concentrations (100 μM versus 20 μM) have been used, since the amounts of α-TOH (100 μM) used in all other experimental setups presented here interfered with the Griess assay through a turbidity of the supernatant that made photometric measurement of the colored reagent N-alpha-naphthyl-ethylenediamine impossible. Optimization of the Griess assay revealed 20 μM of α-TOH as a feasible concentration. Since α-TOH is less efficient in blocking nitric oxide formation compared to other forms of vitamin E (Fig. 3E), the structure of the chromanol ring system seems to be important [3, 28, 29]. Interestingly, the short-chain metabolites α-CEHC and γ-CEHC are also more potent in inhibiting nitric oxide production compared to α-TOH [30]. The α-TOH analogue trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which differs from α-CEHC in length and oxidation of the carboxylate tail, is ineffective in inhibiting nitric oxide formation in EOC-20 cells [30]. Since α-13′-COOH significantly blocks the LPS-induced release of PG almost to baseline levels (Fig. 3), we conclude that the oxidative modification of the side-chain is as important for the inhibition as the chromanol ring.

A closer look to the literature reveals contradictory reports on inhibitory effects of α-TOH on the release of PGE_2 which may depend on the cell type [28, 29, 37]. Studies of non-α-TOH forms and metabolites are more consistent with respect to the inhibition of PGE_2 production [14, 28, 29, 38]. Traber et al. already postulated that the ability of α-TOH metabolites to block the synthesis of PG may depend on the length of the phytyl side chain [39]. Hence, middle and short-chain metabolites of α-TOH were highly effective in blocking the synthesis of PGE_2 probably by inhibition of phospholipase A_2 [39, 40]. Jiang et al. showed that δ-13′-COOH and γ-13′-COOH inhibit the COX2-catalyzed production of PGE_2 in IL1β-stimulated human lung adenocarcinoma A549 cells [14]. We show for the first time that the inhibition of the LPS-stimulated release of nitric oxide and PG by α-13′-COOH is mediated by downregulation of iNos and Cox2, respectively, at the mRNA and protein level. At present, we do not know how the oxidation of the side-chain of α-13′-COOH mediates the observed effects, but our study provides clear evidence for the regulatory features of this metabolite.

Inflammatory pathways are strongly regulated by NFκB that mediates its effects by binding to promoter sites following translocation of the p50/p65 heterodimer from the cytosol into the nucleus [41]. Treatment with α-TOH has been shown to inhibit the activity of NFκB depending on the cell type [28, 42–44]. Using murine RAW264.7 macrophages, we found enhanced LPS-induced translocation of the NFκB subunit p65 after treatment with α-TOH (Fig. 4A). Protein kinase C (PKC) phosphorylates IκB that results in detachment of IκB from the p50/p65 heterodimer and finally in the translocation of the p50/p65 heterodimer into the nucleus. It has been shown that NFκB is inactivated via PKC inhibition by α-TOH in vascular smooth muscle cells [45]. Since our results do not confirm this, other regulatory pathways may mediate the effects of α-TOH on NFκB in RAW264.7 macrophages. Since we investigated only the translocation of the p65 subunit of NFκB into the nucleus, we cannot comment on NFκB activity. As regulation of NFκB activity by metabolites and derivatives of α-TOH, namely 2,2,5,7,8-pentamethyl-6-hydroxychromane and α-tocopheryl succinate, has been described [44], further studies are required to unravel the molecular modes of action of α-13′-COOH. However, these

results again provide evidence that the regulatory pathways affected by α-13′-COOH are different from that of α-TOH.

The α-13′-COOH concentration of 5 μM used here, is relatively high compared to concentrations recently found in vivo [9]. Although α-13′-COOH was found in human serum only in concentrations up to 10 nM [9], we cannot exclude that micromolar amounts occur locally, since significant differences in intra- and extracellular concentrations of metabolites are well known. For example, the α-LCMs were found only in relatively low concentrations in the cytoplasm of liver cells, but concentrations in organelles, such as microsomes, peroxisomes and mitochondria, are much higher [4]. We can therefore expect much higher concentrations, possibly up to the lower micromolar scale, at the tissue or cellular level. Furthermore, in proof-of-principle studies often higher concentrations of bioactive molecules are used in vitro as they are likely available physiologically, as is shown by studies on the regulatory features of oxysterols [46, 47], prostaglandins, and glitazones [48], as well as short-chain metabolites of α-TOH [30]. At present, we do not know the cellular concentrations of the LCMs that can be achieved in vitro and in vivo so that uptake experiments have to be performed in future studies to better understand the physiological relevance of the findings outlined here.

To sum up, our findings clearly demonstrate that α-13′-COOH modulates the inflammatory response of macrophages more potently and via signaling pathways likely different from that of α-TOH. Thus, we present here further hints for a physiological role of the metabolite α-13′-COOH. Finally, our data show that the mode of action of α-TOH may be much more complex due to the regulatory features of its metabolite α-13′-COOH. Further investigations are therefore required to elucidate the in vivo relevance of our findings.

We thank Waltraud Scheiding and Maria Braun for their excellent technical assistance. The project was funded to S.L. by grants from Forschungskreis der Ernährungsindustrie (FEI) as part of an AiF (Arbeitsgemeinschaft industrieller Forschungsvereinigungen "Otto von Guericke") project of the Industrielle Gemeinschaftsforschung (IGF) and Thüringer Ministerium für Bildung, Wissenschaft und Kultur, Stiftung für Technologie und Forschung. Garcinia kola seeds were a kind gift from AnalytiCon Discovery (Potsdam, Germany). S.L. also receives funding from the DFG RTG1715.

The authors have declared no conflict of interest.

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6.4 Manuskript 4

Long-chain metabolites of vitamin E: interference with lipotoxicity via lipid droplet associated protein PLIN2

Running Title: Long-chain metabolites of vitamin E inhibit lipotoxicity via PLIN2

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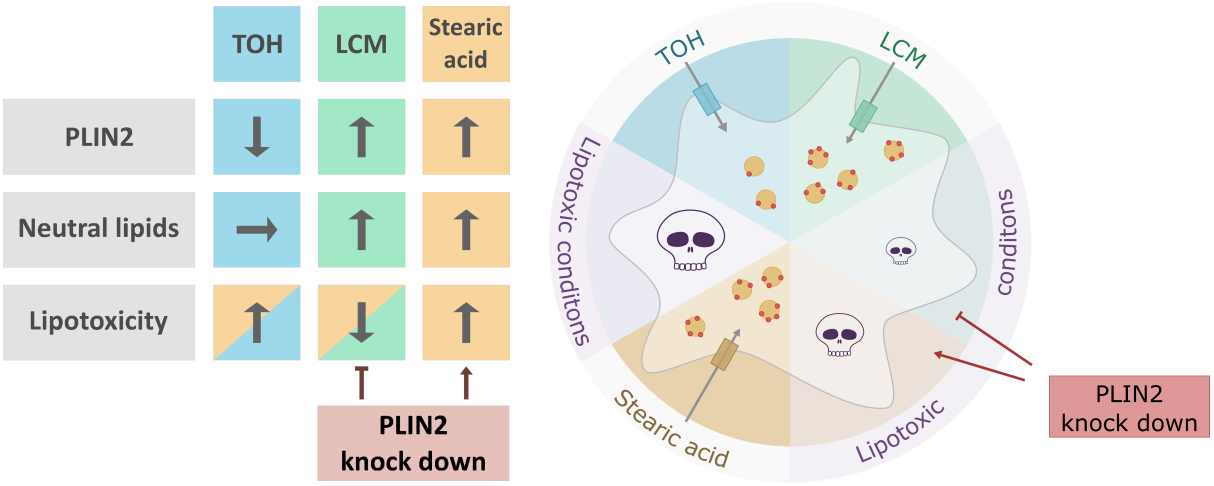
Keywords:

Vitamin E, long-chain metabolites of vitamin E, α-13'-hydroxychromanol, α-13'-OH, α-13'-carboxychromanol, α-13'-COOH

List of nonstandard abbreviations:

α-13'-OH, α-13'-hydroxychromanol, **α-13'-COOH,** α-13'-carboxychromanol, **LCM,** long-chain metabolites of vitamin E, **TOH,** tocopherol, **T3,** tocotrienols

Graphical abstract



Abstract

The long-chain metabolites of vitamin E (LCM) emerge as a new class of regulatory metabolites and have been considered as the active compounds formed during vitamin E metabolism. The bioactivity of the LCM is comparable to the already established role of other fat-soluble vitamins. The biological modes of action of the LCM are far from being unraveled, but first insights pointed to distinct effects and suggested a specific receptor, which in turn lead to the aforementioned hypothesis. Here, a new facet on the interaction of LCM with foam cell formation of THP-1 macrophages is presented. We found a reduced mRNA and protein expression of lipid droplet associated protein PLIN2 by α-tocopherol (α-TOH), whereas the LCM and the saturated fatty acid, stearic acid increased expression levels of PLIN2. In a lipotoxic setup (0 to 800 µM stearic acid and 0 to 100 µM α-TOH or 0 to 2.5 µM α-13'-COOH) differences in cellular viability were found. A reduced viability was observed for cells under co-treatment of α-TOH and stearic acid, whereas an increased viability for stearic acid incubation in combination with α-13'-COOH was measured. The striking similarity of PLIN2 expression levels and impaired or improved lipotoxicity, respectively, revealed a protective effect of PLIN2 on basal stearic acid-induced lipotoxic conditions in PLIN2 knockdown experiments. Based on our results, we conclude that α-13'-COOH protects cells from lipotoxicity at least partly via PLIN2 regulation.

Herewith another facet of LCM functionality was presented and their reputation as regulatory metabolites was further enhanced.

Introduction

Recently, a new perspective on vitamin E and its metabolism was postulated [1]. The metabolites, which are formed during hepatic degradation of tocopherols and tocotrienols, were thought to be just products of vitamin E excess. However, nowadays a change of this paradigm suggests that in particular the long-chain metabolites (LCM), the first metabolites occurring in vitamin E metabolism, may be the activated and thereby functional molecules in the family of vitamin E derivatives. This concept is attractive, as it has already been accepted for other fat soluble vitamins, such as vitamin A or D [2,3].

Vitamin E is a collective term summarizing eight highly similar structures, all of which consist of a chromanol ring-system and an aliphatic side-chain. However, the different forms of vitamin E differ in the saturation of their side-chain, leading to the saturated tocopherols (TOH) and the unsaturated tocotrienols (T3). The methylation pattern of the chromanol ring-system determines the α-, β-, γ- or δ-forms. The hepatic metabolism of vitamin E is principally independent of these features, whereas its efficiency highly depends on the type of methylation (α-TOH is the form with the lowest catabolic rate [4]). In more detail, an oxidative modification of the side-chain via cytochrome P450-dependent enzymes (CYP4F2/CYP3A4) leads to the formation of the LCM, which are in the case of α-TOH, α-13'-hydroxychromanol (α-13'-OH) and α-13'-carboxychromanol (α-13'-COOH). A more detailed overview is provided in a recent review [4].

The biological action of the LCM is far from being unraveled. However, several comprehensive studies have been published over the last years, mainly covering the topics of interaction of LCM with inflammation [5-9], cancer [10,11], handling of pharmaceuticals [12], and macrophage foam cell formation [13], which is a hallmark in the progression of atherosclerosis. In brief, macrophages within the arterial wall are loaded with lipids, mainly originating from oxidized lipoprotein particles (*e.g.*, low density lipoproteins (LDL); for more details, the reader is referred to [14]). These lipids can be stored in so-called cytosolic lipid droplets. Through the light microscope, a cell filled with lipid droplets appears to be foamy, which was eponymous for the foam cells. The lipid droplets are organelles composed of a phospholipid monolayer, a lipid core containing triglycerides and sterol esters, as well as proteins, which are integrated in the phospholipid monolayer [15]. One of these proteins is PLIN2 (formerly adipophilin or adipose differentiation related protein (ADRP)), which was first identified by Jiang *et al.* in 1992 [16,17].

When the lipid loading capacity of cells is exceeded, a mechanism called lipotoxicity is induced [18]. The resulting impaired cellular signaling as well as mitochondrial and ER dysfunction may lead to cell death [19], which in the case of atherogenesis causes the formation of the necrotic lipid core of atherosclerotic plaques. In the initial phase of lipotoxicity, the storage of lipids in lipid droplets is protective as free fatty acids are esterified to triglycerides and are thus removed from active signaling [18,20].

To get a deeper insight into the biological actions of the LCM, we focused on the regulatory effects of α-13'-COOH on the expression of PLIN2, its interference with stearic acid-induced lipotoxicity and the possible connections between both mechanisms.

Materials and Methods

Chemicals

If not indicated otherwise, chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), Fisher Scientific (Schwerte, Germany), or Merck Millipore (Darmstadt, Germany).

Cell culture

THP-1 monocytes (ATCC, Manassas, VA), cultivated in RPMI 1640 supplemented with 10% (v/v) FBS and 0.1 mg/ml penicillin/streptomycin/L-glutamine [21] were differentiated into macrophages using 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 50 µM β-mercaptoethanol [22]. After 96 h, macrophages were incubated with serum-free supplemented medium and the test compounds as indicated in the figures and harvested for further processing as described below.

Incubation

Stearic acid (C18:0; Alfa Aesar, Haverhill, MA) was dissolved in pure ethanol and complexed to fatty acid-free bovine serum albumin (Sigma-Aldrich) at a molar ratio of 4:1 in Krebs-Ringer bicarbonate buffer. α-TOH and LCM were dissolved in DMSO. For incubation, the compounds were mixed with supplemented RPMI 1640 medium without serum in the concentrations indicated in the figures.

Cytotoxicity

THP-1 macrophages were incubated with the respective test compounds in 24-well (standard) or 48-well (transfection) plates; at the end of the incubation period, the cells were washed twice with serum-free supplemented medium. The treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/ml in PBS, 50 µl or 25 µl per well, respectively) in 500 µl or 250 µl serum-free supplemented medium was performed for 4 h. Then the medium was exchanged by 1 ml or 0.5 ml isopropanol and was thoroughly mixed for 10 min, before a centrifugation step (5 min, 300 × g, room temperature) was applied. The solutions were aliquoted (in quadruplicates or triplicates of each 100 µl) to a 96-well plate. Absorption was measured at 570 nm. Viability was calculated by setting the untreated control to 100%.

Concentrations for cell culture studies

The concentrations of the compounds were determined by absorption measurement in pure ethanol. The wavelengths and attenuation coefficients used are 292 nm and ε = 3060 for α-13'-OH and α-13'-COOH.

RNA isolation and cDNA synthesis

Total RNA was isolated using Qiagen RNeasy Mini kit (Hilden, Germany) as described [23]. cDNA synthesis was performed using Revert Aid First strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and 500 ng/µl oligo-dT primers as described [24].

Quantitative real-time RT-PCR (RT-qPCR)

RT-qPCR was run on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Schwerte, Germany) as described [24,25]. Primers (PLIN2, RPL37A, Supplementary Table S1) were purchased from Invitrogen (Karlsruhe, Germany). PCR results were analyzed using the LightCycler software version 1.5.0.39.

Transfection

Transfection of THP-1 macrophages was performed as described by Maeß *et al.* 2014 [26] with slight modifications. Cells were differentiated for 24 h using 100 ng/ml PMA. Transfection was performed using $3 * 10^6$ cells, 1% human serum (Sigma-Aldrich), 2 µg siRNA (PLIN2 Stealth siRNA ADFP HSS174700, 5288746 or Stealth RNAi Negative Control Low GC, 12935200; Thermo Fisher Scientific) and mouse T cell nucleofector medium (Lonza, Basel, Switzerland). Transfected cells were seeded in 48-well plates using 200 µl cultivation medium to prefill wells and 100 µl of cell suspension were added to each well. The incubation with test compounds was started 72 h after transfection.

Immunoblotting

Cells were harvested using a non-denaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 15 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) and samples were processed for Western blotting as described earlier [5]. The proteins were separated by SDS-PAGE and transferred to PVDF membrane (VWR, Darmstadt, Germany). Primary antibodies against PLIN2 (mouse anti-ADRP AP125, 1:50) and α-tubulin (mouse anti-α-tubulin clone B-5-1-2, 1:5000) were purchased from PROGEN (Heidelberg, Germany) and Sigma-Aldrich, respectively. Secondary antibodies (rabbit anti-mouse labeled with horseradish peroxidase, 1:5000) from DAKO (Hamburg, Germany) were used. SignalBoost™ Immunreaction Enhancer Kit (Calbiochem, Darmstadt, Germany) was used for enhancing chemoluminescence signals for PLIN2.

Flow cytometry to measure neutral lipids via nile red

After incubation, cells were detached by Accutase I treatment (Sigma-Aldrich). Following washing steps with PBS, cells were stained with nile red solution (1 µg/ml concentration on cells), incubated for 10 min and washed again. A flow cytometric analysis was performed for neutral lipids in a range of 570 to 590 nm.

Isolation of garcinoic acid and semi-synthesis of α-LCM

Garcinia kola seeds were a gift from AnalytiCon Discovery (Potsdam, Germany). Isolation of garcinoic acid from the African bitter nut *Garcinia kola* and syntheses of the LCM were performed as described [11,27]. Purity of all LCM used was higher than 95%, as confirmed by HPLC-MS.

Statistics

Data are presented either as means ± standard deviation or as means ± standard error of the mean (SEM) of independent experiments as indicated. In order to test for statistical significance, paired Student's t-tests were performed using Microsoft Excel 2010.

Results

The LCM of vitamin E emerged as regulatory metabolites with distinct and specific effects [28]. The main goal of the present study was to enlighten another aspect of their biological activity and to strengthen the new perspective on LCM as 'activated' or 'executive' metabolites, similar to the metabolites of vitamin A or D.

It has been reported previously that the LCM modulate foam cell formation in THP-1 macrophages, among others by inducing the expression of CD36 [13]. Using these experiments as a starting point, the present study focused on the regulation of another lipid metabolism-related protein, namely adipophilin (adipose differentiation related protein, ADRP or PLIN2) and the impact of the LCM on saturated fatty acid-induced lipotoxicity, as an important event in lipid-driven diseases [19].

PLIN2 is a lipid droplet-associated protein. Based on studies showing effects of vitamin E on lipid-related proteins and lipid metabolism, we investigated the effect of α-TOH and its metabolites on PLIN2. We found that PLIN2 expression was significantly reduced by α-TOH on mRNA and protein level after 24 h by 47% to 26%, respectively ($p < 0.01$; Fig. 1). The LCM, however, induced PLIN2 expression on both levels in a range of 1.6- to 3.8-fold ($p < 0.05$). The expression of PLIN2 is known to be induced by fatty acids, such as stearic acid. Hence, we were interested in the regulation of PLIN2 under stearic acid and LCM treatment. Therefore, THP-1 macrophages were pre-treated with α-TOH or the respective LCM for 24 h, followed by a co-incubation with stearic acid for additional 24 h before protein expression analysis via Western blot (Fig. 2). For both pre-treatments (α-TOH and α-13'-COOH), the co-incubation with stearic acids resulted in expression values similar to that of stearic acid alone. Similar results were achieved by neutral lipid staining of cells using nile red. The cellular levels of neutral lipids were not influenced by α-TOH, but were induced 1.5- to 1.8-fold by α-13'-COOH and stearic acid, respectively. The co-incubation with α-TOH and α-13'-COOH did not affect the stearic acid-induced accumulation of neutral lipids.

However, stearic acid is known to induce lipotoxicity, when the concentrations applied exceed the cell's capacity of saturated fatty acid handling. We pictured this by incubating the cells with stearic acid in concentrations up to 800 µM. Thereby, the treatment with a concentration range for α-TOH (0 µM to 100 µM) or α-13'-COOH (0 µM to 2.5 µM) made the impact of LCM on lipotoxicity obvious (Fig. 3). While α-TOH impaired the stearic acid-induced lipotoxicity (300 µM stearic acid: viability of control cells 88%, viability of cells treated with 100 µM α-TOH: 77%), α-13'-COOH partly protected the cells, *e.g.* for the treatment with 600 µM stearic acid

an improvement of cell viability by α-13'-COOH of 13% to 15% was found.

Keeping in mind that the LCM can induce PLIN2, we wondered whether the reduced lipotoxicity of stearic acid by the LCM is mediated via PLIN2. Therefore, knockdown studies of PLIN2 were performed and the impact of its knockdown on stearic acid-induced lipotoxicity was tested. The knockdown was followed by an incubation regime similar to the approach used for Fig. 3 (incubation with 0 µM to 800 µM stearic acid). It was very clear that the cells treated with siRNA against PLIN2 were more prone to the lipotoxic effect of stearic acid than the controls (16% decreased viability by PLIN2 knockdown for 400 µM stearic acid; $p < 0.01$; Fig. 4).

To study the contribution of the PLIN2 knockdown to the protective effect of the LCM on stearic acid-induced lipotoxicity, the transfected cells were pre-incubated with the α-13'-COOH for 24 h and different concentrations of stearic acid were applied for further 24 h (Fig. 5). The effective stearic acid concentration was calculated for several viabilities (40% to 85%) for both transfections (control siRNA and PLIN2 siRNA) and these were plotted *vs.* the respective LCM concentration. The slope for the defined viabilities across the LCM concentration was calculated and these were averaged within the siRNA treatments. A significant difference between these slopes was found (siCTRL: grey line, siPLIN2: dotted line; $p < 0.001$). This means that although a protective effect of α-13'-COOH is still observed under PLIN2 knockdown, it is by far less pronounced than in cells transfected with control siRNA. Therefore, it can be assumed that α-13'-COOH protects from stearic acid-induced lipotoxicity at least partly via the regulation of PLIN2 protein levels.

Discussion and Conclusions

Recent studies on the metabolism of vitamin E revealed the physiological presence of the LCM α-13'-OH and α-13'-COOH in human blood [6,13]. We therefore hypothesize that the LCM are available at the site of action, *e.g.* in the case of atherogenesis at intimal macrophages. A recent study provided convincing evidence for the specific and distinct signaling mediated by the LCM even at concentrations lower than their precursors [28]. Based on these results, a specific, not yet identified receptor for LCM was proposed. To elucidate the biological and molecular mechanisms of the LCM in more detail, a study on the regulatory effect of LCM on the scavenger receptor CD36 [13] was used as a starting point for the experiments described here. We aimed to getting a deeper insight into the regulation of foam cell formation by the LCM and focused on the regulation of PLIN2 at basal conditions and under load with the saturated stearic acid.

The expression of the lipid droplet-associated protein PLIN2 was assessed in human THP-1 macrophages after α-TOH and α-LCM treatment under basal conditions, *i.e.* incubation without serum. To the best of our knowledge, we are the first to describe the inhibition of PLIN2 expression by α-TOH and its induction by the α-LCM on mRNA and protein level. The neutral lipid accumulation precisely followed the regulation of PLIN2, as measured after incubation of 48 h by nile red staining and subsequent flow cytometric analysis. Due to delayed

accumulation of neutral lipids (no significant effects after 24 h (data not shown), but prominent accumulation after 48 h; Fig. 2), we conclude that the LCM induce PLIN2 expression, which in turn leads to lipid accumulation. This is likely as PLIN2 is known to elevate cellular lipid levels by inhibiting β-oxidation [29] and lipolysis [30]. Thus, the lipid storage capacity of the cells is increased by the LCM via induced PLIN2 expression, followed by increases in neutral lipid accumulation.

In contrast to the aforementioned experiments, we focused on experiments under fatty acid stimulus. While stearic acid incubation led to the expected increase in PLIN2 expression [31] on protein level and in neutral lipid accumulation [32], the co-incubation with stearic acid and α-13'-COOH showed no additive effect (Fig. 2). This can be explained by mechanisms each specific for LCM and stearic acid. The readout (PLIN2 expression and lipid accumulation) may lead to the same results, but the underlying mechanisms are likely distinct. For stearic acid, a direct interaction with the PLIN2 protein via specific binding pockets has been described [33], while analogous studies are pending for the LCM. However, it is not yet established whether this influences neutral lipid accumulation or PLIN2 expression. Each mechanism may also be regulated or influenced by the respective other compound, which in turn may lead to the non-additive effect seen under the co-treatment setup. Next, another important aspect might be the heterogeneity of lipid droplets [34,35], which could be influenced by the LCM. Lipid droplets vary not only in size or contact sites to other organelles, *e.g.* ER [36], but also in lipid [35] and protein [37] composition. Interestingly, PLIN2 is localized on lipid droplets of all sizes (starting with pre-lipid droplets) [36,37]. Interestingly, a protective social organization of cells has been observed while handling lipotoxicity [38]. A subpopulation of cells sacrifices themselves by excessive lipid storage to moderate the lipotoxic conditions for the community. Keeping in mind the heterogeneity of lipid droplets and cellular lipid handling, one could imagine a distinct effect mediated by compounds such as LCM, *e.g.* a specific channeling of exogenous fatty acids (here, stearic acid) and endogenous fatty acids (induction of lipid accumulation by the LCM under serum-free conditions) to distinct lipid droplets.

We focused on the interaction of α-TOH and LCM with stearic acid-induced lipotoxicity. For this purpose, cells were treated with a compound-concentration-matrix of stearic acid (0 to 800 µM) and α-TOH (0 to 100 µM) or α-13'-COOH (0 to 2.5 µM), respectively. Stearic acid-induced lipotoxicity, measured by the MTT viability test, has been described [39]. Here, we describe for the first time that stearic acid-induced lipotoxicity was impaired by α-TOH, whereas α-13'-COOH reduced the effect of stearic acid. So far, neither the effects of vitamin E nor its metabolites on lipotoxicity have been investigated, thus the underlying mechanisms are an object of speculation. However, a clear analogy to the study designed by Rabkin *et. al.* [39] is striking. Rabkin also induced lipotoxicity via stearic acid, but oleic acid (C18:1) was used for rescuing cardiomyocytes from death. Surprisingly, the intracellular lipid pattern measured under co-incubation of stearic acid and oleic acid was similar to our results for α-13'-COOH in neutral lipid accumulation, which is a non-additive effect. Keeping in mind the low similarity in their structure, the similar findings for the LCM and oleic acid are unexpected.

We would like to point the reader's attention to the impact of fatty acid distribution to PLIN2 binding on the surface and size of lipid droplets. The binding affinity of PLIN2 for oleic acid is twice the affinity of stearic acid [40] and introducing saturated acyl chains to the phospholipids of the monolayer forming the surface of lipid droplets impairs PLIN2 binding due to the

condensation of the phospholipid monolayer covering lipid droplets [41]. However, larger intracellular lipid droplets have a higher proportion of saturated fatty acids in their monolayer than smaller ones [42]. A closer look into the fatty acid profile of cells treated with the LCM might enlighten the contribution of the aforementioned mechanisms to the results described here.

The effect of α-TOH or LCM on stearic acid-induced lipotoxicity is accompanied with the regulation of PLIN2 expression. Therefore, we asked whether PLIN2 might be the crux of the matter and set up knockdown experiments for PLIN2. At basal conditions (stearic acid concentration gradient only, no incubation with α-13'-COOH), an impairment of stearic acid-induced lipotoxicity was found, which is likely due to a loss in lipid storage capacity forced by the knockdown of PLIN2. Knockdown of PLIN2 has been described to influence cellular lipid levels and size of lipid droplets as well as their number [29,43]. It has also been suggested that PLIN2 plays a role in the expansion of lipid droplet size [44]. To sum up, this could lead to the impairment of stearic acid-induced lipotoxicity by the knockdown of PLIN2. However, it has been described that other homologs of PLIN2, *e.g.* TIP47 [45] take on its task if PLIN2 is knocked down. This might be the reason why the worsening of stearic acid-induced lipotoxicity by the knockdown of PLIN2 is relatively mild. Taking together, PLIN2 is protective against stearic acid-induced lipotoxicity to some extent.

A cross-comparison of the respective cell viabilities between Figure 3 and 4 highlights the increased sensitivity of transfected cells towards stearic acid treatment. It is known that electroporation (the transfection method used here) is able to transfer lipids between membrane leaflets [46], which may induce further perturbations in lipid metabolism or handling. Overall this might contribute to the higher sensitivity of transfected cells to mediators of lipotoxicity.

Next, the stearic acid lipotoxicity tests were conducted in the presence of the knockdown of PLIN2 and LCM. While the LCM-dependent blocking of lipotoxicity was observed under both conditions (control and PLIN2 knockdown), the effective stearic acid concentrations needed to achieve a certain viability, were significantly reduced by the knockdown of PLIN2 across all α-13'-COOH concentrations tested. This leads to two conclusions: (i) PLIN2 expression protects macrophages from stearic acid-induced lipotoxicity, and (ii) the LCM protect macrophages from lipotoxicity partly via the induction of PLIN2. It might be striking that the reduction in lipotoxicity by α-13'-COOH seems not to be coupled with an additive induction of neutral lipid accumulation. On the other hand, it has been shown that the cellular lipid content is not a determinant of lipotoxicity-induced cell death [39].

Overall, we have made several interesting new observations: First, PLIN2 protects macrophages form stearic acid-induced lipotoxicity. Second, α-TOH triggers stearic acid-induced lipotoxicity in macrophages. Third, we found evidence for a complex link between the α-LCM, PLIN2 and lipotoxicity. We provide first evidence for the potentially protective effects of the α-LCM by inducing lipid storage capacity and thereby decreasing the proneness to lipotoxicity. Out data also show that PLIN2 contributes at least partly to this phenomenon. Paving in paths for mechanistic studies underlying this first descriptive study on this topic will contribute to the growing knowledge on the modes of action of the LCM, which we consider as

a new class of regulatory metabolites.

Acknowledgements

The work of S.L. is supported by grants from the Federal Ministry of Education and Research (01EA1411A), the Deutsche Forschungsgemeinschaft (DFG; RTG 1715) and the German Ministry of Economics and Technology (AiF 16642 BR) via AiF (German Federation of Industrial Research Associations), FEI (Research Association of the German Food Industry) and by the Free State of Thuringia and the European Social Fund (2016 FGR 0045). The work of L.S. is supported by the Free State of Thuringia and the European Social Fund (2016 FGR 0045). The work of M.W. is also funded by the DFG (Wa 3836/1-1). We thank Waltraud Scheiding, Maria Braun and Alexander Maxones for their excellent technical assistance.

Conflict of interest statement

The authors declare no competing interests.

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Figures

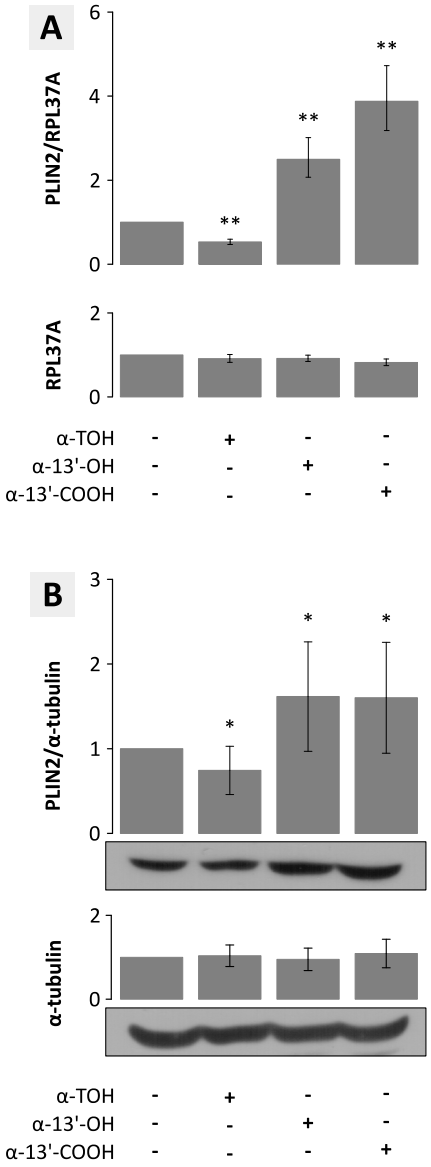

Figure 1
PLIN2 expression is induced by α-LCM but reduced by α-TOH
Human THP-1 macrophages were incubated with 100 µM α-TOH, 10 µM α-13'-OH or 5 µM α-13'-COOH for 24 h and processed for mRNA **(A)** or protein **(B)** expression analysis. Expression of PLIN2 was reduced under α-TOH treatment by 47 % (SEM min 7 %, SEM max 6 %) on mRNA level and by 26 % ± 28 % on protein level. In contrast, the LCM α-13'-OH induced the PLIN2 expression on mRNA level by 250 % (SEM min 43 %, SEM max 51%) and 162 % ± 65 % on protein level. More pronounced effects were obvious with α-13'-COOH: an induction of mRNA expression by 388 % (SEM min 69 %, SEM max 85 %) and of protein expression by 160 % ± 65 % was found. **(A)** PLIN2 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions. Error bars display calculated maximum and minimum expression levels of mean expression levels of three independent biological experiments each measured twice. **(B)** PLIN2 protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions. Images of Western blot analyses show representative results. Mean expression levels of four independent biological experiments each measured twice are shown. *, $p < 0.05$; **, $p < 0.01$ (*vs.* control).

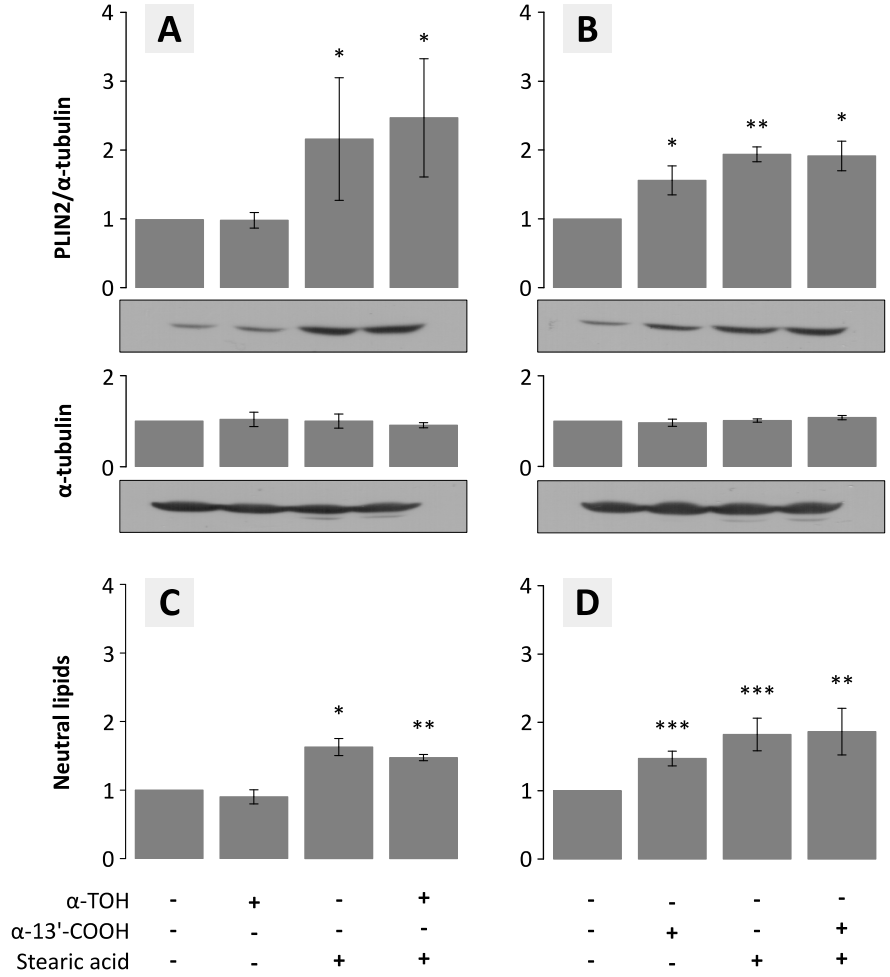

Figure 2
PLIN2 expression and neutral lipid accumulation is induced by stearic acid and α-13'-COOH

Human THP-1 macrophages were incubated with 100 µM α-TOH **(A + C)** or 5 µM **(B)** or 2.5 µM **(D)** α-13'-COOH for 24 h and with 150 µM stearic acid for additional 24 h in the presence or absence of the compounds. Afterwards, cells were processed for protein expression analysis via Western blot **(A+B)** or neutral lipid staining **(C+D)**.

(A) While α-TOH did not regulate PLIN2 expression on the protein level, stearic acid-induced PLIN2 by 216 % ± 66 % and the combinatory incubation resulted in an induction of 222 % ± 67 %. **(B)** The LCM α-13'-COOH induced the PLIN2 protein expression by 156 % ± 21 %, while stearic acid-induced its expression by 194 % ± 11 %. In combination, both compounds induced PLIN2 protein expression by 191 % ± 22 %. PLIN2 protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions. Images of Western blot analyses show representative results. Mean expression levels of four **(A)** or three **(B)** independent biological experiments each measured once are shown.

(C) While α-TOH did not regulate neutral lipid accumulation, stearic acid-induced neutral lipids by 163 % ± 13 % and the combinatory incubation resulted in an induction of 147 % ± 5 %. **(D)** α-13'-COOH induced neutral lipid accumulation by 147 % ± 11 %, while stearic acid-induced neutral lipid accumulation by 182 % ± 24 %. In combination, both compounds induced neutral lipid accumulation by 186 % ± 34 %. Neutral lipid accumulation was calculated relatively with the control set to 1. Mean expression levels of three **(C)** or five **(D)** independent biological experiments each measured once are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*vs.* control).

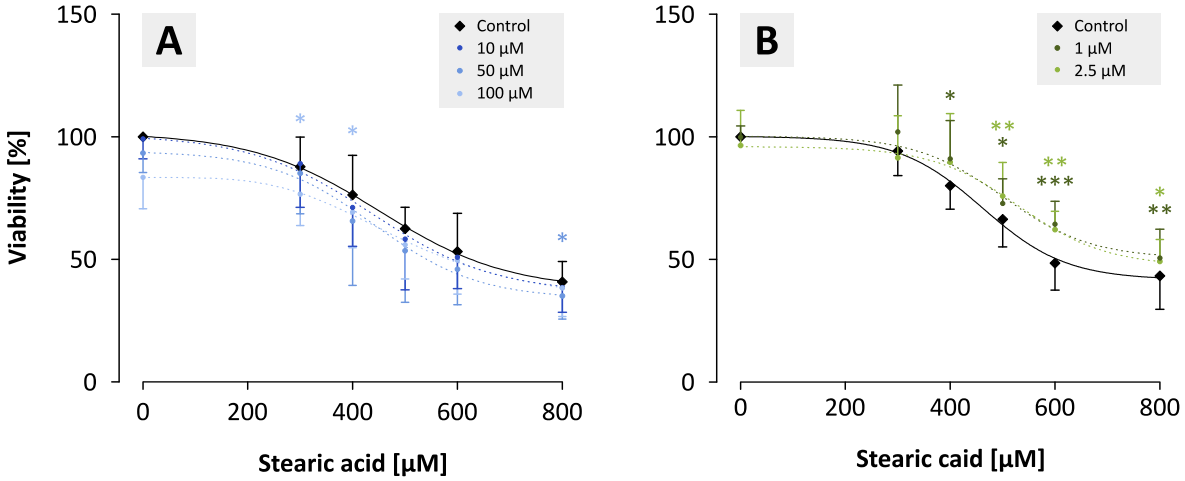

Figure 3

Stearic acid-induced lipotoxicity is partly reduced by α-13'-COOH but not by α-TOH

Human THP-1 macrophages were incubated with 0 to 100 µM α-TOH **(A)** or 0 to 2.5 µM α-13'-COOH **(B)** for 24 h, followed by a co-incubation with 0 to 800 µM stearic acid for further 24 h. This incubation matrix was assessed by MTT cytotoxicity tests. **(A)** Treatment of THP-1 macrophages with increasing concentrations of stearic acid resulted in reduced cell viability. The same holds true for cells, which were co-treated with α-TOH, whereas the viability seems to be further reduced for some concentrations (300 µM stearic acid: viability of control cells 87.8 % ± 12 % *vs*. viability of cells treated with 100 µM α-TOH: 76.7 % ± 12.9 %, $p < 0,05$). **(B)** Again, the concentration-dependent reduction of viability under stearic acid treatment was seen. When cells were co-incubated with α-13'-COOH the viability of the cells was significantly increased (600 µM stearic acid: 48.5 % ± 11.0 % in controls *vs.* 64.4 % ± 9.4 % in cells treated with 1 µM α-13'-COOH or 62.2 % ± 7.4 % in cells treated with 2.5 µM α-13'-COOH, respectively; $p < 0.001$ or $p < 0.01$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*vs*. control).

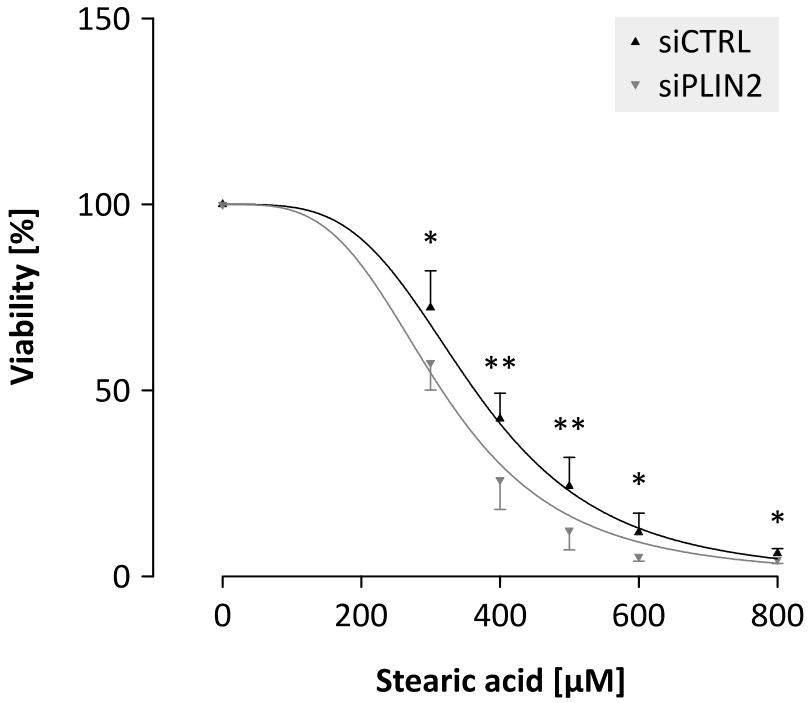

Figure 4

PLIN2 partly protects from stearic acid-induced lipotoxicity

Human THP-1 macrophages were transfected with control siRNA (siCTRL) or PLIN2 siRNA (siPLIN2) and incubated with increasing concentrations of stearic acid (0 to 800 µM) for 24 h. Afterwards, the cells were treated with MTT solution and the readout was performed as described in the Materials and Methods section. With increasing concentrations of stearic acid the viability of control cells decreased (300 µM stearic acid: 72.26 % ± 9.9 % viability, 500 µM stearic acid: 24.21 % ± 5.1 % viability). Cells treated with PLIN2 siRNA were more prone to stearic acid-induced lipotoxicity (300 µM stearic acid: 57.47 % ± 7.4 % viability, $p < 0.05$ (control *vs.* PLIN2 siRNA treatment), 500 µM stearic acid: 12.39 % ± 5.3 % viability, $p < 0.01$ (control *vs.* PLIN2 siRNA treatment)). Error bars display standard deviations of mean viability levels of four independent biological experiments each measured once. *, $p < 0.05$; **, $p < 0.01$ (*vs.* control).

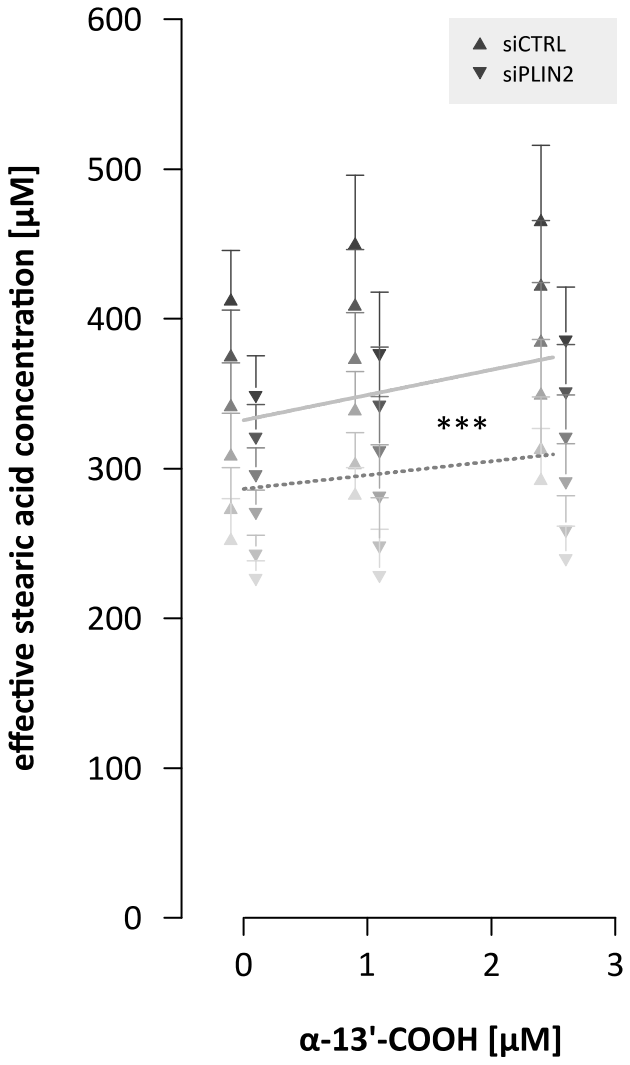

Figure 5
α-13'-COOH protects from stearic acid-induced lipotoxicity partly via PLIN2
Human THP-1 macrophages were transfected with control siRNA (siCRTL) and PLIN2 siRNA (siPLIN2) and incubated with 0 to 2.5 µM α-13'-COOH and 0 to 800 µM stearic acid. A plot showing the dependency of the viability from stearic acid concentration was calculated for each LCM concentration. A logarithmic fit was performed, and the effective concentrations of stearic acid for 40 % to 85 % viability were calculated. For each viability, the slope for the concentration dependency of LCMs on the effective concentration of stearic acid was calculated. The mean for each effective concentration of stearic acid was obtained from four biological replicates. Finally, the mean was calculated across all experiments, which were treated with the same siRNA. The comparison of both slopes, siCRTL (grey line) *vs.* siPLIN2 (dotted line) in dependency of LCM concentration and the effective concentration of stearic acid revealed a significant difference (***, $p < 0.001$).

Supporting Material

Long-chain metabolites of vitamin E: interference with lipotoxicity via lipid droplet associated protein PLIN2

Running Title: Long-chain metabolites of vitamin E inhibit lipotoxicity via PLIN2

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Table S1: PCR primers used in this study. In each case, forward and reverse primers are located in different exons.

mRNA	mRNA name	Origin	GenBank accession no.	Forward primer	Reverse primer	Amplicon size [bp]
PLIN2	Adipose differentiation related protein	Human	NM_001122	CTGATGAGTCCCACTGTGCTGA	TGTGGCACGTGGTCTGGAG	90
RPL37A	Ribosomal protein L37a	Human	NM_000998	ATTGAAATCAGCCAGCACGC	AGGAACCACAGTGCCAGATCC	94

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Redox Biology 2 (2014) 495–503

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Graphical Review

Regulatory metabolites of vitamin E and their putative relevance for atherogenesis

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ARTICLE INFO

Article history:
Received 29 December 2013
Received in revised form
7 February 2014
Accepted 11 February 2014
Available online 19 February 2014

Keywords:
α-Tocopherol
α-Tocopherol long-chain metabolites
α-13′-COOH
Atherosclerosis
Macrophage foam cells

ABSTRACT

Vitamin E is likely the most important antioxidant in the human diet and α-tocopherol is the most active isomer. α-Tocopherol exhibits anti-oxidative capacity *in vitro*, and inhibits oxidation of LDL. Beside this, α-tocopherol shows anti-inflammatory activity and modulates expression of proteins involved in uptake, transport and degradation of tocopherols, as well as the uptake, storage and export of lipids such as cholesterol. Despite promising anti-atherogenic features *in vitro*, vitamin E failed to be atheroprotective in clinical trials in humans. Recent studies highlight the importance of long-chain metabolites of α-tocopherol, which are formed as catabolic intermediate products in the liver and occur in human plasma. These metabolites modulate inflammatory processes and macrophage foam cell formation via mechanisms different than that of their metabolic precursor α-tocopherol and at lower concentrations. Here we summarize the controversial role of vitamin E as a preventive agent against atherosclerosis and point the attention to recent findings that highlight a role of these long-chain metabolites of vitamin E as a proposed new class of regulatory metabolites. We speculate that the metabolites contribute to physiological as well as pathophysiological processes.

Contents

Introduction

Atherosclerosis is a progressive inflammatory disease characterized by excessive deposition of cholesterol in the arterial wall. Despite intensive therapeutic treatment opportunities the atherosclerotic complications are still the leading cause of death in Western industrialized countries.

Leonardo da Vinci (1452–1519) was probably the first who described the macroscopic changes of atherosclerosis, when he illustrated the lesions in arteries obtained from an elderly man at autopsy. His visionary idea was that the pathological thickening

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http://dx.doi.org/10.1016/j.redox.2014.02.002

Nomenclature

α-13′-OH	α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol
α-13′-COOH	α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid
α-CEHC	α-carboxyethyl-hydroxychroman
α-LCM	α-tocopherol long-chain metabolites
α-SCM	α-tocopherol short-chain metabolites
α-TOH	α-tocopherol
COX	cyclooxygenase
CYP3A4	cytochrome P450, subfamily IIIA, polypeptide 4
CYP4F2	cytochrome P450, subfamily IVF, polypeptide 2
LDL	low density lipoprotein
oxLDL	oxidized low density lipoprotein

of the arterial wall was due to 'excessive nourishment' from the blood. Many decades later da Vinci's observation was studied in more detail by Carl von Rokitansky (1852) and Rudolf L. K. Virchow (1821–1902). In 1856 Virchow proposed that injury of the endothelium may initiate the disease process of atherosclerosis. Based on this idea, Russell Ross (1929–1999) and John A. Glomset came up in 1973 with the 'response-to-injury' hypothesis which is still generally accepted today in the form of the more generalized concept of endothelial dysfunction as the initial cause of atherosclerosis.

The pioneering work of Virchow and Nikolai N. Anitschkow (1885–1964) provided first evidence for the importance of the deposition of lipids from the blood, in particular cholesterol, in the arterial wall. Their findings formed the basis for the lipid hypothesis which connects plasma cholesterol levels to the development of the disease. In 1951, G. Lyman Duff (1904–1956) and Gardner C. McMillan (1918–2004) formulated the lipid hypothesis in its modern form, which is, despite controversial discussions, still widely accepted today. Since the discovery of the importance of the cholesterol contained in low-density lipoprotein (LDL[2]) particles for the pathogenesis of atherosclerosis, the concept of endothelial dysfunction has become tightly linked to the lipid hypothesis.

Almost 30 years ago the concept originated from work by Daniel Steinberg and Joseph L. Witztum that oxidative stress and the oxidation of LDL particles might contribute to atherosclerosis. The idea came up from the observation that the incubation of macrophages with oxidized LDL (oxLDL[3]) but not with native LDL led to the intracellular accumulation of cholesteryl esters. The idea that oxidative stress is involved in atherogenesis gained much attention and created tremendous excitement to look for oxLDL *in vivo* as well as for different kinds of oxidized lipid species within the particle. Since oxLDL appears in human plasma as well as within the arterial wall it was even a small step to the idea that supplementation with antioxidants may prevent atherosclerosis by inhibiting the formation of oxLDL. This hypothesis appeared to be on solid ground due to epidemiological evidence and the success in several animal studies using a variety of antioxidants. The euphoria of initial success led to clinical trials to validate the hypothesis and natural antioxidants were of particular interest as the expectation was that these natural compounds would have less undesirable effects. Accordingly a number of clinical trials were performed using, for example, vitamin E, which surprisingly have not been overwhelmingly supportive of the hypothesis. An overview on the controversial findings for vitamin E obtained from clinical trials is given in Fig. 1.

In this review, we want to summarize the controversial role of vitamin E as a preventive agent against atherosclerosis and to point the attention to recent findings by our group that highlight a role of long-chain metabolites of vitamin E as a proposed new class of regulatory metabolites and to their potential contribution to atherosclerotic processes.

[2] LDL, low density lipoprotein.
[3] oxLDL, oxidized low density lipoprotein.

Pathogenesis of atherosclerosis

The endothelium covering the arterial walls comprises a physiological and selective barrier, the so-called intima, between blood and the inner layer of the arterial wall. This so-called media is comprised by contractile smooth muscle cells. Pathophysiological stimuli cause endothelial dysfunction triggering inflammatory processes in the vascular wall which result under chronic conditions in extensive morphological changes characterized by intimal thickening, deposition of cholesterol and fibrotic material, loss of elasticity, reduction of vascular lumen, and widening of the vessel diameter [1]. Endothelial dysfunction is thought to be caused by exogenous stimuli, such as environmental factors (e.g., toxicants such as dioxins, PCBs, and pesticides), unhealthy lifestyle (e.g., smoking and physical inactivity) and dietary habits (e.g., high intake of saturated fat). The impact of exogenous factors depends on endogenous local and systemic conditions. Local factors are vessel-associated junctions, bifurcations and curvatures which are responsible for increased shear stress caused by turbulences of the blood stream in these areas, which are thus predestinated for the formation of atherosclerotic lesions [2]. Proatherogenic systemic factors are determined either genetically or pathophysiologically, for example, in case of increased LDL and triglyceride plasma levels [3,4] as well as inflammatory conditions [5]. The process of atherosclerosis is outlined and explained in more detail in Fig. 2.

A key event of atherogenesis is the loss of the selective endothelial barrier by endothelial dysfunction which allows, for example, LDL to enter the arterial wall. Once inside the vessel wall, LDL particles become prone to oxidation. The oxidized particles cause damage to the tissue thus triggering a cascade of immune and inflammatory responses. In addition, macrophages, the phagocytic cells of the immune system, are recruited to the affected tissue sites to clear the oxLDL particles. As a consequence oxidized lipids and particularly cholesterol accumulate within the macrophages as these cells are not able to process the oxLDL completely. This causes transformation of the cells into so-called foam cells and ultimately cell death as the excessive accumulation of intracellular lipids is cytotoxic. Death of macrophage foam cells results over time in the extracellular deposition of cholesterol in the arterial wall and the formation of an atheroma. The process of atherosclerosis is outlined and explained in more detail in Fig. 2.

Thus, vitamin E was considered as an anti-atherogenic agent for a long time as prevention of LDL oxidation by providing increased levels of antioxidants would prevent the formation of macrophage foam cells and atheroma, and would dampen the immune and inflammatory response.

Effects of α-Tocopherol on atherogenic processes

Vitamin E is likely the most important lipid antioxidant in the human diet. The term vitamin E comprises a group of eight abundant isomers (α-, β-, γ-, δ-tocopherol and -tocotrienol), that differ by their methylation patterns of the hydroxychromanol ring and saturation of the side-chain. Many *in vitro* studies have been performed with

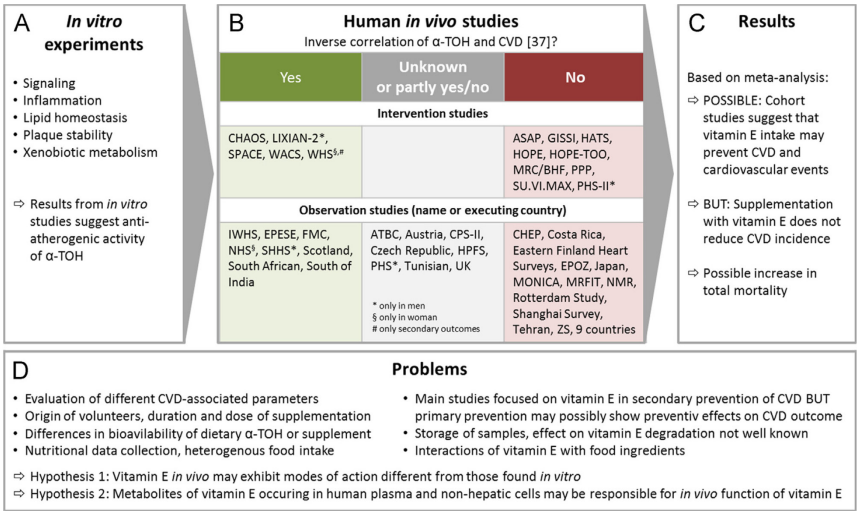

Fig. 1. Effects of α-TOH *in vivo*. Many *in vitro* investigations (A) focused on the identification of α-TOH-regulated signaling pathways and its effects on inflammation, lipid homeostasis, atherosclerotic plaque stability as well as xenobiotic metabolism as key processes. Taken together most of the *in vitro* studies implicated that vitamin E, and particularly α-TOH, may be used to prevent or cure cardiovascular disease (CVD) and related diseases, such as atherosclerosis. Based on very promising studies *in vitro* and with animals several large-scaled human intervention trials were initiated and followed up over years. Unfortunately, the trials revealed controversial results and failed to demonstrate clear invers relations or positive effects of α-TOH supplementation with respect to the prevention of cardiovascular complications [21] (B). Further, α-TOH serum levels did not correlate with cardiovascular outcomes in different cohorts. Although some studies reported promising findings, such as the Nurses' Health Study (NHS) [22] including 87,000 volunteers in which vitamin E supplementation was associated with a lower risk of major coronary disease, other large-scale studies, such as the Heart Outcomes Prevention Evaluation (HOPE) study [23], the SU.VI.MAX study [24], the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) study [25] did not confirm that vitamin E intake correlates negatively with cardiovascular outcomes. Other studies revealed also contrary results depending on the time of follow-up and the cardiovascular parameter investigated or they showed unclear results (for example, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) [26–30], the Physicians' Health Study [31], the Health Professionals Follow-up Study (HPFS) [32,33]). In a recent meta-analysis Ye et al. also found no significant inverse correlation for CVD and cardiovascular mortality under vitamin E [34]. Reasons for the poor outcomes of clinical trials are hardly to define. Beside a general failure of vitamin E, other reasons may explain the lack of any cardio-preventive effect. Likely the selection of volunteers, the sizes of cohorts, doses and duration of supplementation or rather the application form of vitamin E with respect to its bioavailability, the food questionnaires and variability of food intake may explain the findings. Furthermore, it cannot be excluded that the interaction of vitamin E with other food ingredients contributes to the lack of its cardio-preventive activity. From a technical point of view, it cannot be excluded that vitamin E in stored sample is chemically modified or degraded so that frozen biological samples are not completely comparable to fresh samples. Another important point refers to the fact that most of the intervention trials concentrated on secondary prevention in patients with already existing CVD. It has been suggested that vitamin E supplementation may be more effectively for inhibiting the early stages of atherosclerosis [35] and should be considered for primary prevention, as recently supported by Meydani et al. emphasizing the beneficial effects of long-term vitamin E supplementation in $Ldlr^{-/-}$ mice under healthy life-style conditions, such as low fat diet [36]. However, this hypothesis has not yet been confirmed. In summary (C), there is no clear evidence that supplementation with vitamin E correlates inversely with CVD incidence. Meta-analyses of observation studies suggest that vitamin E intake may prevent CVD and cardiovascular events [37–39]. Knekt et al. performed a pooled analysis of observation studies with dietary vitamin E intake and supplementation in separate arms and found a significant inverse correlation of intake and CVD events only in the supplementation group [40]. Apart from that meta-analysis of intervention studies provide evidence that supplementation with vitamin E does not reduce CVD incidence [38,41–43]. There are several drawbacks of meta-analysis that should be considered while interpreting these results, such as combination of heterogeneous data sets (regarding quality, statistics and focus within the topic), publication bias as well as criteria for inclusion and exclusion of the meta-analysis. However, it cannot yet be excluded that vitamin E intake is protective at least in some groups of humans against CVD as primary prevention. It is also important to remind that Miller et al. focused in their meta-analysis on some intervention studies, which provided evidence for an increase in all-cause mortality after supplementation with high doses of vitamin E [44]. These findings in humans raise the question whether α-TOH *in vivo* exhibits modes of action different from those found *in vitro*. Possible explanations for the inability of vitamin E to prevent CVD and its complications in clinical trials in humans have been outlined above and are summarized in (D). Abbreviations and references: Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) [26–30], Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) [45], Austria [46], Cambridge Heart Antioxidant Study (CHAOS) [47], Cancer Prevention Study II (CPS-II) [48], Costa Rica [49], Czech Republic [50], Eastern Finland Heart Surveys [51], Epidemiologic Study of Cardiovascular Risk Indicators (EPOZ Study) [52], Established Populations for Epidemiologic Studies of the Elderly (EPESE) [53], Finnish Mobile Clinic Examination Survey (FMC) [54], Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico (GISSI) [25], HDL-Atherosclerosis Treatment Study (HATS) [55], Health Check-up Program (CHEP) [56], Heart Outcomes Prevention Evaluation Study (HOPE) [23], HOPE-The Ongoing Outcomes [HOPE-TOO] [57], Iowa Women's Health Study (IWHS) [58], Japan [59], Lixian dysplasia trial (LIXIAN-2) [60,61], Medical Research Council/British Heart Foundation (MRC/BHF) [62], Multinational MONitoring of trends and determinants in CArdiovascular disease (MONICA) [63], Noninstitutionalized Massachusetts Residents (NMR) [64], Physicians' Health Study (PHS) [31], Physicians' Health Study II (PHS-II) [65], Rotterdam Study [66], Scotland and UK [67], Scottish Heart Health Study (SHHS) [68], Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE) [69], Shanghai Survey [70], South African [71], South of India [72], Supplémentation en Vitamines et Minéraux Antioxydants (SU.VI.MAX) [24], Tehran [73], The Health Professionals Follow-up Study (HPFS) [32,33], The Multiple Risk Factor Intervention Trial (MRFIT) [74], The Nurses' Health Study (NHS) [22], The Primary Prevention Project (PPP) [75], The Zutphen Study (ZS) [76], Tunisian [77], UK [78], Women's Antioxidant Cardiovascular Study (WACS) [79], Women's Health Study (WHS) [80], 9 countries [81]. The name of countries/cities refers to the countries/cities in which the studies have been performed, if no name for the studies is available.

α-tocopherol (α-TOH[4]) which is the most active isomer within the group of vitamin E [6]. α-Tocopherol exhibits anti-oxidative capacity *in vitro* [7], and it has been shown to particularly inhibit, for example, the oxidation of LDL. Beside this, α-TOH shows anti-inflammatory features by, for example, inhibiting cyclooxygenase (COX[5]) 2. Next to its anti-inflammatory and anti-oxidative properties, the vitamin E

[4] α-TOH, α-tocopherol.

[5] COX, cyclooxygenase.

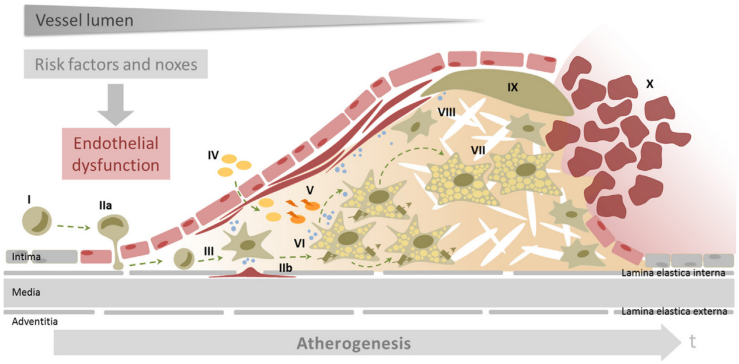

Fig. 2. Key events of atherogenesis. Atherosclerosis is a complex and progressive inflammatory disease characterized by extensive morphological changes of the vascular wall. The arterial wall is composed of the *intima*, formed by endothelial cells (EC), the *media*, comprised of smooth muscle cells (SMC), and the *adventitia*, made of fibroblasts. The intermediate layers of the arterial vessel are the *lamina elastica interna*, connecting the *intima* with the *media*, and the *lamina elastica externa*, the connection between the *media* and the *adventitia*. Key changes of the vascular wall during the progression of atherosclerosis are intimal thickening, extensive extracellular deposition of cholesterol and fibrotic material, loss of elasticity, reduction of the vascular lumen and widening of the arterial diameter [1]. Endothelial dysfunction is caused by exogenous and endogenous noxes and is the initial event in atherosclerosis. The dysfunction of the endothelium is accompanied by up-regulation of adhesion molecules in EC, which promote attachment and recruitment of T-lymphocytes and monocytes from the blood (I) and initiate immigration of monocytes into the subendothelial area of the vessel wall (IIa). Migration of monocytes, T-lymphocytes and SMC from the *media* (IIb) finally results in intimal thickening and fibrosis. Following migration of monocytes through the *intima*, differentiation of these cells into macrophages occurs (III). Macrophages as the phagocytic cells of the immune system play a pivotal role in the progression of atherosclerosis. They actively engage by taking up lipids from oxLDL in a non-controlled manner, by storing large amounts of cholesteryl esters, and by mobilizing cholesterol for reverse cholesterol transport [82,83]. Macrophages also orchestrate the inflammatory process, are responsible for the immigration of SMC from the *media* by releasing chemotactic molecules and proteases, and modulate the fibrotic process. LDL particles diffuse from the blood into the subendothelial space as a consequence of the loss of the endothelial barrier during endothelial dysfunction (IV). In the arterial wall the lipids and LDL are subjected to oxidation and enzymatic modification. The resulting oxLDL is taken up by macrophages (V) via scavenger receptors and phagocytosis in uncontrolled fashion [84]. Recent studies have also highlighted the intra-plaque proliferation of macrophages in the lipid-rich stage of atherosclerotic plaque development (VI) [85]. Accumulation and uptake of oxLDL by macrophages triggers the secretion of chemotactic molecules [86], which promote the migration of SMC from the *media* into the subendothelial tissue. The intimal SMC lose their ability to contract, proliferate and synthesize extracellular matrix which results in fibrosis as part of the plaque development. The deposition of the extracellular matrix leads to further accumulation of oxLDL and lipids, in particular cholesterol and cholesteryl esters. The uptake of oxLDL via scavenger receptors is not subjected to negative feedback regulation, thus resulting in excessive intracellular lipid accumulation and formation of macrophage foam cells (VII) [87], as well as the release of chemotactic mediators. During further progression of atherosclerosis, fatty streaks are formed and the thickening of the vascular wall progresses through the ongoing deposition of extracellular lipids and proliferation of intimal SMC, and the accompanied synthesis of extracellular matrix proteins. Over many years these processes together form the characteristic necrotic lipid core covered and stabilized by a fibrotic cap (VIII). The stability of the atherosclerotic plaque is defined by the amount of accumulated lipids and also by the amount and quality of the extracellular matrix of the fibrotic cap; the progressing accumulation of lipids is often accompanied by reduced stability of the fibrotic cap (IX). Weakening of the cap may finally result in rupturing of the plaque, particularly in areas rich in macrophages as these cells produce a broad range of proteases that degrade the extracellular matrix of the fibrotic cap [88,89]. Plaque rupture causes thrombus formation (X) via the activation of the coagulation cascade [87]. Thrombi may occlude the artery at the site of plaque rupture or may flow through the blood stream and occlude downstream arteries that have a smaller lumen. In some cases, thrombi at the plaque site are reorganized and integrated into the plaque. This finally leads to the formation of the so-called complicated plaque (not shown).

isomers may have a variety of further independent properties, namely the modulation of gene expression, particularly that of genes encoding proteins involved in signaling but also the uptake, transport and degradation of tocopherols, as well as the uptake of lipoproteins and the storage and export of lipids such as cholesterol. The *in vitro* and *ex vivo* effects of α-tocopherol on cellular processes are depicted in Fig. 3.

α-Tocopherol metabolites and their bioactivity

Metabolic degradation of α-TOH takes place almost exclusively in the liver. Beside metabolites resulting from oxidation of the chroman moiety, hepatic metabolism of α-TOH involves CYP3A4[6]-dependent ω-hydroxylation and α-oxidation, which results in the formation of the α-tocopherol long-chain metabolites (α-LCM[7]) α-13′-OH[8] (13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol) and α-13′-COOH[9] (13′-(6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-yl)-2,6,10-trimethyl-tridecanoic acid), and further steps of β-oxidation, which results in the formation of middle- and short-chain metabolites (α-SCM[10]) with the catabolic end-product α-carboxyethyl-hydroxychroman (α-CEHC[11]), respectively [8–10]. The short-chain metabolites are excreted via urine and are often used as a marker for α-TOH supply [11]. Other tocopherols, such as γ- and δ-tocopherol, are almost quantitatively degraded and excreted via the urine as the corresponding γ- and δ-CEHCs. The hepatic metabolism of α-TOH is illustrated in Fig. 4.

Regulatory activity is not restricted to α-TOH as its short-chain metabolite α-CEHC also exhibits bioactivity. It has been shown that α-CEHC is anti-proliferative [12], anti-inflammatory [13], and anti-oxidative [14], and inhibits oxLDL formation [15] and protein kinase C (PKC[12]) signaling [16]. Recently, researchers focused also on investigating the cellular effects of the α-LCM as α-13′-COOH was detected in human serum, a finding providing clear evidence for its systemic bioavailability. Until now, only a few cellular effects of the α-LCM have been described, such as pro-apoptotic, anti-proliferative and anti-inflammatory features [17–20], which are highlighted in Fig. 4.

[6] CYP3A4, cytochrome P450, subfamily IIIA, polypeptide 4.
[7] α-LCM, α-tocopherol long-chain metabolite(s).
[8] α-13′-OH, α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol.
[9] α-13′-COOH, α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid.
[10] α-SCM, α-tocopherol short-chain metabolite(s).
[11] α-CEHC, α-carboxyethyl-hydroxychroman.
[12] PKC, protein kinase C.

Endothelial cells			
Signaling	⇩ PKC	⇩ NFκB activation	⇧ PPARγ DNA binding
Inflammatory process	⇩ Migration	⇩ Glucose-induced IL-8 release	⇧ Catalase expression
	⇩ Stimulus-induced VCAM-1, ICAM-1, E-selectin	⇩ H_2O_2-induced lipid peroxidation	⇩ Intracellular ROS production
	⇩ Monocyte adhesion to ECs	⇧ H_2O_2 degradation activity	⇩ Apoptosis by scavenging ROS
	⇩ Platelet adhesion on ECs	⇧ Intracellular GSH level	⇩ Apoptosis by inhibiting caspase activity
	⇧ eNOS activity		
Monocytes and macrophages			
Lipid homeostasis	⇩ CD36	⇩ ABCA1/ABCG1	
	⇩ SR-A	⇩ oxLDL-induced ABCA1/G1 expression	
Signaling	⇩ NFκB activation		
Inflammatory process	⇩ Migration	⇩ Stimulus-induced COX-2 and iNOS expression as well as PGE_2 and NO release	⇩ Stimulus-induced release of IL-1β, IL-6, TNF-α, IFN-γ, MCP-1 and IL-8
	⇩ Stimulus-induced adhesion		
	⇩ Stimulus-induced CD11b, VCAM-1, VLA-4	⇩ 7-Ketocholesterol induced apoptosis	⇩ Superoxide anion release
Smooth muscle cells			
Lipid homeostasis	⇩ CD36	⇩ oxLDL uptake	
Signaling	⇩ PKC	⇩ PMA-induced ERK activation	⇩ Akt-PKB dephosphorylation
	⇧ Protein phosphatase 2A	⇧ DAG kinase activation	
Inflammatory process	⇩ Migration	⇩ SMC proliferation by PKC inhibition	⇩ 7-Ketocholesterol induced apoptosis
Plaque stability	⇧ CTGF (connective tissue growth factor, stimulates synthesis of extracellular matrix) expression		
Hepatocytes			
Lipid homeostasis	⇩ CD36	⇩ Isopentenyl-diphosphate δ isomerase	⇩ Squalene synthase
	⇩ LDL-R	⇩ Farnesyl diphosphate synthase	⇧ 27-Hydroxycholesterol under high C-diet
	⇩ HMG-CoA reductase and synthase	⇩ 7-Dehydrocholesterol reductase	⇧ CYP27A1 under high C-diet
Signaling	⇩ NFκB activation		
Inflammatory process	⇩ Stimulus enhanced iNOS expression		
xenobiotic metabolism	⇧ CYP3A11 to levels which might interfere with drug metabolism		
Non-cellular			
Plaque stability	⇧ Increased number of plaques with thicker cap	⇩ Percentage of ruptured plaques	⇩ oxLDL formation
	⇩ Necrotic core areas	⇩ MMP3 expression	⇩ Long-term
			⇩/⇧ Short- term: depending on condition

Fig. 3. Atherosclerosis-relevant *in vitro* and *ex vivo* effects of α-tocopherol. Due to its function as an antioxidant, vitamin E was considered to interfere with key events in atherogenesis. To gain better insights into the contribution of vitamin E to the molecular processes underlying the hallmarks of atherosclerosis much effort was spend on *in vitro* and *ex vivo* experiments as well as studies involving animal models. As the complex pathogenesis of atherosclerosis involves several different cell types, the figure is divided according to the cells of interest (EC, SMC, monocytes and macrophages, hepatocytes) as well as non-cellular, plaque-specific processes and categories such as lipid homeostasis, signaling, inflammation, plaque stability and xenobiotic metabolism which are used to reflect the hallmarks of atherosclerosis. Endothelial cells surface the arterial wall and their dysfunction is the initial step of atherogenesis. α-Tocopherol reduces the stimulus-induced expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin [90–92], and decreases thereby the adhesion [92] and immigration of leukocytes [93] onto the endothelium or into the arterial wall, respectively. α-Tocopherol makes it easier for EC to deal with oxidative stress, such as H_2O_2-induced lipid peroxidation [7], due to higher catalase expression [94], increased H_2O_2 degradation activity, and higher intracellular GSH levels [13]. Thus, apoptosis of EC induced by oxidative stress is reduced [95]. Beside reducing the release of inflammatory cytokines, such as interleukin (IL) 8 [96], in response to external stimuli, α-TOH interferes with cellular signaling by inhibiting, for example, PKC [97], activate nuclear factor κB (NFκB) [98] or binding of peroxisome proliferator-activated receptor γ (PPARγ) to its regulatory DNA elements [94]. Lipid homeostasis of SMC is also affected by α-TOH, for example by down-regulation of the scavenger receptor CD36 leading to reduced uptake of oxLDL [99]. α-Tocopherol inhibits migration [100] and proliferation of SMC by inhibiting PKC [101], and prevents 7-ketocholesterol-induced apoptosis [102]. Plaque stability is modulated by connective tissue growth factor (CTGF), which stimulates the synthesis of extracellular matrix. This factor is induced by α-TOH in SMC [103], suggesting that α-TOH contributes to plaque stability by inducing fibrotic processes. Several signaling cascades in SMC are also modulated by α-TOH, namely PKC [104,105], extracellular signal-regulated kinase (ERK) [106], protein kinase B (PK/AKT) and protein phosphatase 2A [107]. Monocytes migrate in the arterial wall, differentiate into macrophages and transform to foam cells under atherogenic conditions. α-Tocopherol reduces adhesion and migration of leukocytes by down-regulating expression of CD11b [93] and very late antigen-4 (VLA-4) [92,108]. Macrophage foam cell formation is prevented by reducing the expression of scavenger receptors CD36 [109,110] and A [111]. On the other hand, foam cell formation is triggered by the down-regulation of the lipid exporters ATP-binding cassette transporter (ABC) A1 and G1 [110]. Furthermore, the inflammatory response of macrophages to stimuli such as lipopolysaccharide (LPS) is dampened. The diminished induction of COX2 and inducible nitric oxide synthase (iNOS) by α-TOH results in a reduced release of prostaglandin E_2 (PGE_2) and nitric oxide [112,113]. The release of pro-inflammatory cytokines such as IL-1b, IL-6, tumor necrosis factor α (TNFα), and interferon γ (IFNγ) is also reduced [113]. Similar to SMC, 7-ketocholesterol-induced apoptosis is reduced by α-TOH in macrophages [114,115]. Signaling pathways, such as NFκB [108] and oxLDL or lipid-free high density lipoprotein induced liver X receptor α (LXRα) activity, are also inhibited by α-TOH [110]. The liver is the major organ for cholesterol biosynthesis and metabolism of lipoproteins, xenobiotics and α-TOH. In liver cells α-TOH down-regulates expression of CD36 [116,117] and the LDL receptor [118], as well as expression of enzymes involved in cholesterol biosynthesis, such as HMG-CoA reductase and HMG-CoA synthase [118]. In contrast, expression of cytochrome P450 subfamily 27A polypeptide 1 (CYP27A1), and thus synthesis of 27-hydroxycholesterol, is induced by α-TOH [119]. Similar to the situation in macrophages, activation of NFκB and expression of iNOS in response to stimuli is reduced by α-TOH [120]. α-Tocopherol also induces CYP3A11 to levels which might interfere with drug metabolism [121]. In addition to the effects of α-TOH on the different cell types involved in atherogenesis, α-TOH improves plaque stability in hyperlipidemic rabbits. Treatment of the animals with α-TOH increased the number of plaques with thicker, stabilizing fibrotic caps and reduced necrotic lipid core areas as well as reduced number of ruptured plaques [122]. Formation of oxLDL was also blocked by α-TOH [123]. Several studies using animals such as hypercholesterolemic rabbits or mice suggest that vitamin E inhibits atherogenesis in early [124] or advanced stages by its antioxidant capacity [125] or gene regulatory potential via signal transduction cascades and on adhesion molecules [126,127]. While high dose supplementation of vitamin E can improve myocardial tolerance to ischemia and reperfusion [128], Keaney et al. found that low dose α-TOH improves and high dose worsens endothelial vasodilatory function in cholesterol-fed rabbits [129]. Under extreme conditions, such as pronounced elevation in systemic oxidative stress due to hyperlipidaemia and obesity, vitamin E seems to be not cardioprotective [130].

A recent study by our group showed that α-LCM also affect macrophage foam cell formation by regulating uptake of oxLDL by macrophages via down-regulation of its phagocytic uptake (Fig. 4) [17]. A key finding of our study was that bioactivity of the α-LCMs occurs at much lower concentrations and with mechanisms distinct from those of their metabolic precursor α-TOH.

Perspective

The findings obtained from clinical trials with humans raise the question whether vitamin E *in vivo* exhibits modes of action different from those found *in vitro*. Recent studies shed new light on mechanistic aspects of α-TOH function, which appear to be complicated by

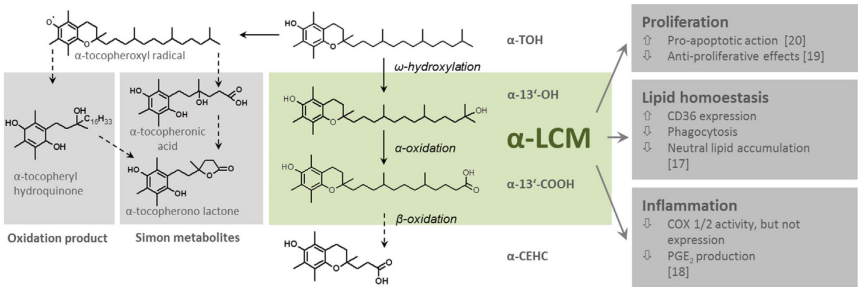

Fig. 4. α-Tocopherol metabolism and *in vitro* characteristics of its liver-derived long-chain metabolites. Due to the antioxidant capacity of vitamin E, early studies on its metabolism have concentrated on metabolites resulting from oxidation of the chroman moiety. The major oxidation product in the liver was described as α-tocopheryl quinone. This metabolite derives from the reaction of the tocopheroxyl radical with a peroxyl radical; it can be reduced to α-tocopheryl hydroquinone by NAD(P)H-dependent microsomal and mitochondrial enzymes. For many years, the so-called Simon metabolites, α-tocopheronic acid and its lactone, were the only known urinary α-TOH metabolites. The Simon metabolites are characterized by the opened chroman ring. Opening of the chroman ring starts with the formation of an α-tocopheroxyl radical when α-TOH has exerted its antioxidant activity. The Simon metabolites were therefore considered as urinary indicators that α-TOH had reacted as an antioxidant [131]. Today some researcher rise the question whether Simon metabolites are artefacts produced during sample preparation as α-CEHC is easily converted to α-tocopheronolactone by oxygenation [10,132]. α-Tocopherol is physiologically catabolized in the liver via the xenobiotic detoxification system involving CYP3A4 [8] and CYP4F2[133]. Cytochrome-dependent ω-hydroxylation results in the formation of the long-chain alcohol derivate α-13′-OH, 13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol. Subsequent α-oxidation leads to α-13′-COOH, 13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid. The following β-oxidation steps in peroxisomes and mitochondria form the α-SCM, α-carboxyethyl-hydroxychroman (α-CEHC) [6]. This end-product of α-TOH metabolism can be conjugated and is excreted via urine [134]. The intact chroman structure indicates that α-CEHC is derived from α-TOH that has not reacted as an antioxidant. As α-CEHC excretion increases when certain plasma levels of *RRR*-α-TOH are exceeded, excretion of α-CEHC is considered as an indicator of adequate or excessive α-TOH supply. Although hepatic metabolism of α-TOH and the formation of the metabolic long- and short-chain intermediates are known for several years [8–10], the physiological function of the α-LCM α-13′-OH and α-13′-COOH is still unknown. Due to a lack of the pure compounds α-13′-OH and α-13′-COOH, only a few studies on the function of these α-LCM have been performed. Work so far focused on anti-proliferative effects, modulation of inflammatory processes and modulation of lipid homeostasis. Our group described anti-proliferative effects of the α-LCM due to pro-apoptotic action [20]. In HepG2 cells, the α-LCM induced cleavage of caspases 3, 7 and 9 as well as PARP-1 and induced mitochondrial dysfunction as characterized by reduced mitochondrial membrane potential and induced intra-mitochondrial ROS formation. The anti-proliferative effect of α-13′-COOH was shown also in murine glioma C6 cancer cells [19]. Others have reported that the α-LCM interfere with inflammatory processes by modulating activity of COX1 and COX2 and consequently by blocking production of PGE_2 [18]. Recent work by our group focused on the effects of the α-LCM on macrophage foam cell formation [17]. We have shown that α-LCM induce expression of the scavenger receptor CD36, the major receptor responsible for oxLDL uptake, in human macrophages, in contrast to the inhibiting actions of α-TOH [17]. Despite up-regulation of CD36, uptake of oxLDL and oxLDL-induced lipid accumulation was reduced in human macrophages, similar to the effects of α-TOH on oxLDL-mediated foam cell formation. An important finding of this recent study was that the metabolite α-13′-COOH was detected in serum providing for the first time evidence for the bioavailability of the α-LCM outside the liver. Another key finding of the study was that bioactivity of the α-LCM occur at lower concentrations and with mechanisms distinct from those of α-TOH. Taken together, these recent studies provide evidence for a role of the α-LCM as signaling molecules derived metabolically from α-TOH.

α-LCM circulating in the blood. We speculate that the α-LCM represent a new class of regulatory metabolites and propose that unraveling the molecular modes of action of the α-LCM and identifying the key players involved in their signaling may provide new fundamental insights into the biology and mode of function of vitamin E. Further studies are therefore required to elucidate the physiological role of the α-LCM and their contribution to disease processes, such as atherosclerosis. We also hypothesize that the discrepancy between the results obtained *in vitro* and *in vivo* in humans may be due to the physiologic metabolism of α-TOH and the formation of α-LCM in the liver and their release into circulation.

Sources and Funding

Research of SL is funded by Grants from the *Forschungskreis der Ernährungsindustrie* (FEI) as part of the *Arbeitsgemeinschaft industrieller Forschungsvereinigungen* (AiF) and the *Deutsche Forschungsgemeinschaft* (DFG).

Disclosures

None.

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DOI: 10.4331/wjbc.v7.i1.14

World J Biol Chem 2016 February 26; 7(1): 14-43
ISSN 1949-8454 (online)

REVIEW

Complexity of vitamin E metabolism

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Author contributions: Schmölz L, Birringer M, Lorkowski S and Wallert M drafted parts of the manuscript; Birringer M and Lorkowski S revised it critically; Lorkowski S and Wallert M contributed equally.

Supported by Grants from "Forschung für die Praxis "of the Hessisches Ministerium für Wissenschaft und Kunst to Birringer M; and grants from the Federal Ministry of Education and Research and the Deutsche Forschungsgemeinschaft to Lorkowski S as an acknowledgement to the national institutions that currently support our research in the field of vitamin E and its long-chain metabolites.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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Received: August 24, 2015
Peer-review started: August 27, 2015
First decision: October 27, 2015
Revised: November 25, 2015
Accepted: January 16, 2016
Article in press: January 19, 2016
Published online: February 26, 2016

Abstract

Bioavailability of vitamin E is influenced by several factors, most are highlighted in this review. While gender, age and genetic constitution influence vitamin E bioavailability but cannot be modified, life-style and intake of vitamin E can be. Numerous factors must be taken into account however, *i.e.*, when vitamin E is orally administrated, the food matrix may contain competing nutrients. The complex metabolic processes comprise intestinal absorption, vascular transport, hepatic sorting by intracellular binding proteins, such as the significant α-tocopherol-transfer protein, and hepatic metabolism. The coordinated changes involved in the hepatic metabolism of vitamin E provide an effective physiological pathway to protect tissues against the excessive accumulation of, in particular, non-α-tocopherol forms. Metabolism of vitamin E begins with one cycle of CYP4F2/CYP3A4-dependent ω-hydroxylation followed by five cycles of subsequent β-oxidation, and forms the water-soluble end-product carboxyethylhydroxychroman. All known hepatic metabolites can be conjugated and are excreted, depending on the length of their side-chain, either *via* urine or feces. The physiological handling of vitamin E underlies kinetics which vary between the different vitamin E forms. Here, saturation of the side-chain and also substitution of the chromanol ring system are important. Most of the metabolic reactions and processes that are involved with vitamin E are also shared by other fat soluble vitamins. Influencing interactions with other nutrients such as vitamin K or pharmaceuticals are also covered by this review. All these processes modulate the formation of vitamin E metabolites and their concentrations in tissues and body fluids. Differences in metabolism might be responsible for the discrepancies that have been observed in studies performed *in vivo* and *in vitro* using vitamin E as a

supplement or nutrient. To evaluate individual vitamin E status, the analytical procedures used for detecting and quantifying vitamin E and its metabolites are crucial. The latest methods in analytics are presented.

Key words: Vitamin E metabolism; Long-chain metabolites of vitamin E; Vitamin E bioavailability; Vitamin E transport

Core tip: Several factors influence vitamin E bioavailability. Gender, age and genetic constitution cannot be modified but life-style and vitamin E intake can be. Physiological handling of vitamin E involves intestinal absorption, vascular transport, hepatic sorting by intracellular binding proteins, and hepatic metabolism. These processes involve kinetics which vary between the different vitamin E forms. The coordinated metabolism of vitamin E is an effective physiological pathway to prevent excessive accumulation of non-α-tocopherol forms. Interactions with other nutrients or pharmaceutics occur. To evaluate vitamin E status, analytical procedures to detect and quantify vitamin E and metabolites are crucial. Current state-of-the-art analytics are presented.

Schmölz L, Birringer M, Lorkowski S, Wallert M. Complexity of vitamin E metabolism. *World J Biol Chem* 2016; 7(1): 14-43 Available from: URL: http://www.wjgnet.com/1949-8454/full/v7/i1/14.htm DOI: http://dx.doi.org/10.4331/wjbc.v7.i1.14

INTRODUCTION

Vitamin E is recognized as being essential for human health, yet fundamental questions remain. In the words of Eduardo Cardenas[1]: "This is a very exciting time in vitamin E research, yet it is evident that we are far away from making the final decision on the benefit *vs* risk for the potential use of vitamin E in human health". Underlying this lack of understanding is the immense complexity involved in the metabolism of vitamin E. Despite many decades of research on vitamin E, many relevant processes remain puzzling. In this review we will outline what is known today including more uncertain claims about vitamin E, to deepen insight into the physiological mechanisms and its metabolism.

Evans and Bishop were the first in 1922 to describe the relevance of vitamin E in the reproduction of rats and to characterize tocopherols (TOH) and tocotrienols (T3) including their α-, β-, γ- and δ-forms as vitamins[2]. Vitamin E belongs to the group of fat-soluble vitamins and occurs dominantly in oily plants; therefore, nuts, seeds and oils are good sources for vitamin E. Almonds, hazelnuts, germ oil and sunflower oil contain high amounts of α-TOH while walnuts, palm oil and soybeans predominantly contain γ-TOH[1]. T3 are widely found in some cereals, palm oil and rice bran oil[3]. Coconut oil, cocoa butter, soybeans, barley and wheat germ are also naturally occurring sources of T3s[4], whereas vegetables and fruits - with the exception of dried apricots, some legumes, avocado and green olives - contain lower levels of vitamin E forms[5]. The concentration of vitamin E forms contained in food depends on several factors, such as growing, harvesting and any further processing (refining or cooking)[5,6].

BIOAVAILABILITY

Bioavailability of vitamin E is influenced by numerous factors including: (1) the amount of vitamin E and intake of interfering nutrients; (2) proteins involved in vitamin E absorption and individual differences in the efficiency of vitamin E absorption, influenced by for example diseases; (3) vitamin E metabolism; (4) life-style factors; (5) gender; and (6) genetic polymorphisms. For an overview, see Figure 1.

Vitamin E intake: Recommendations

When considering dietary vitamin E, official intake recommendations are provided by various boards and institutes, and are theoretically defined amounts of a nutrient providing an adequate intake for the major part of a healthy population[7]. Here there exists a subtle difference in definitions describing levels of vitamin intake; whereas vitamin deficiency is caused by diseases or metabolic disorders, vitamin undersupply is characterized as an intake issue which does not achieve reference values[7].

As of today there is no generally accepted recommendation defining the value for an adequate intake of vitamin E. This is due to different references used to validate the recommended dietary allowance (RDA) for vitamin E, or α-TOH. Whereas in the United States the correlation of hydrogen peroxide-induced erythrocyte lysis and plasma α-TOH concentrations is used[8], in Germany, Austria and Switzerland the RDA for vitamin E is based on the effects of vitamin E on the prevention of lipid peroxidation[9]. At present, the German Society of Nutrition (Deutsche Gesellschaft für Ernährung) recommends a daily vitamin E intake of 12 mg/d for women and 13-15 mg/d for men, for both adolescents and adults; intake should be higher during pregnancy (13 mg/d) and breast-feeding (17 mg/d). The required amount of vitamin E increases with age for infants and children and decreases in the elderly independent of gender[10]. Generally, the recommended intake of vitamin E should correlate with the amount of polyunsaturated fatty acids in food: 1 g of diene fatty acid or rather diene equivalent requires an intake of 0.5 mg *RRR*-α-TOH.

Although several foods contain naturally occurring sources of vitamin E, it is frequently the case that the intake recommendations are not achieved. In Germany infants and children up to age twelve commonly do not

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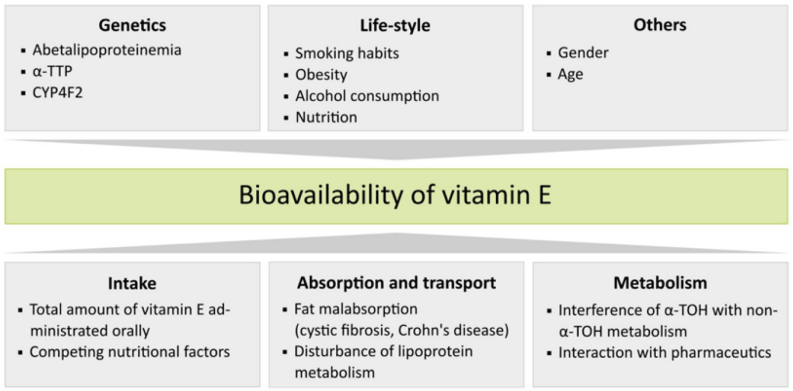

Figure 1 Factors influencing the bioavailability of vitamin E. Some factors affecting bioavailablity cannot be influenced, such as gender, age or genetic disposition, whereas others depend on individual habits and can be summarized as life-style factors. Variations in the physiological handling of vitamin E can also change its distribution status in the body. While uptake of vitamin E can be actively modulated, for example by the total amount of vitamin E intake and competing nutritional factors, absorption and transport principally depend on the state of health of the individual. In contrast, regulation of vitamin E metabolism is just partly influenced by exogenous factors, such as interfering pharmaceuticals.

reach the recommended levels of vitamin E intake[7], as shown in a number of studies including the VELS investigation (Verzehrsstudie zur Ermittlung der Lebensmittelaufnahme von Säuglingen und Kleinkindern; Food Consumption Survey of Babies and Infants) and the EsKiMo study (Ernährungsstudie als KiGGS-Modul, Nutritional Study as KiGGS modul), a follow-up of the KiGGS study (Kinder- und Jugendgesundheitssurvey; Children and Adolescence Health Survey). In addition some studies show that the elderly are often undersupplied with vitamin E[7,11-13]. Numerous research groups analyzing compliance to the vitamin E intake recommendations in Americans have found that a significant number of individuals consume insufficient amounts[14-16]. Dietary intake surveys by the United Kingdom and the United States National Health and Nutrition Examination Survey have revealed that at least 75% of the population consumes vitamin E far below recommended values[17]. Although the recommended amount of vitamin E is higher for men than for women, Dutch women consume less vitamin E more often compared to Dutch men[17]. Data by Traber[18] published last year suggest that more than 90% of United States Americans consume insufficient amounts of vitamin E.

As vitamin E is primarily stored in adipose tissue (about 90% of the body's total vitamin E content[19]), vitamin E deficiency is almost unknown under normal physiological conditions[19]. Symptoms of slight vitamin E undersupply become apparent after many years, usually after decades[19], since vitamin E can be mobilized from adipose tissue for years[20]. In contrast, serious vitamin E deficiency manifests in acute symptoms, such as neuropathy and myopathy, as vitamin E is essential for the development and maintenance of the central nervous system[21]. Ulatowski and Manor[16] categorized metabolic vitamin E deficiencies as: (1) a primary deficiency "(…) arising from specific alteration in vitamin E status"; and (2) a secondary deficiency, "(…) in which low levels of vitamin E are secondary to other global perturbations such as disorders in lipid malabsorption or lipoprotein metabolism and transport". Next to dietary habits, hereditary disorders are known to cause primary and secondary vitamin E deficiencies or inadequate vitamin E bioavailability[19].

Because the official intake recommedations of vitamen E are so seldom met, along with the rare occurance of deficiency symptoms (apart from being caused by disease, addressed later), Traber[18] recently questioned whether the recommended α-TOH intake is set too high and whether a diet low in dietary α-TOH intake has any biological significance. However as of now, circulating α-TOH concentrations below 9 mmol/L for men or below 12 mmol/L for women are considered as deficient and only marginal for healthy adults, respectively[18].

When supplements are used to maintain vitamin E balance, either self-medicated or by medical prescription, questions of toxicity and other safety concerns must be considered. Recent animal studies on reproduction or investigations on the developmental toxic effects of natural or non-natural vitamin E components have been negative but anti-mutagenic activity has been shown[22]. Physiological vitamin E intake of 100 mg/d (150 IU/d) can be increased up to 300 mg/d (450 IU/d) without any complications[23,24]. Even for short-term, high-dose administration of vitamin E no adverse effects have been described[22]. However, persistent high-dose supplementation has been shown to interfere with blood clotting and has been associated with increased risk of hemorrhagic stroke in animal studies[22]. In the past, TOH was considered to be a safe food additive[25]. But, Miller *et al*[26] reported an increase of total mortality

after high-dose vitamin E intake for at least one year. Since adverse effects, such as increased tendency to hemorrhage, have been observed at high vitamin E intake, the tolerable upper intake level for adults was set at 1000 mg/d α-TOH[8].

Life-style and age influence the bioavailability of vitamin E

Concentrations of serum nutrients such as vitamin E are influenced by age and lifestyle factors (*e.g.,* obesity, tobacco smoking, alcohol consumption)[27]. Age-related differences or changes in vitamin E levels in serum and tissue have been discussed in numerous studies. Campbell *et al*[28] showed that vitamin E decreased after the age of 80 years and argued that this finding is possibly connected with reduced food intake of elder people. In contrast, hepatic levels of vitamin E have been found to not be significantly affected by age[29]. Other studies have reported enhanced serum concentrations of vitamin E in the elderly from 60 years[27,30,31], which may be attributed to the age-dependent increases in serum cholesterol/lipoprotein concentrations[30]; this may be protective against increased lipid peroxidation during aging[29,32]. Another explanation was given by Succari *et al*[31], who suggested that life-style and age-associated changes independent of serum cholesterol/lipoprotein concentrations are responsible for the increased vitamin E levels in aged French women.

Numerous clinical trials have demonstrated a gender-independent inverse relationship between human obesity and serum α-TOH concentrations[33,34]. However, clear correlations between serum α-TOH concentrations and general obesity [defined by body mass index (BMI) or body fat percentage] have not been shown, but other parameters for obesity, such as waist circumference and waist-to-hip ratio, were positively associated with α-TOH serum concentrations in both men and women[35-37]. Indeed, Wallström *et al*[35] reported from the Malmö Diet and Cancer Study cohort that serum levels of α-TOH were associated with central adiposity after adjustment for body fat. In contrast, Gunanti *et al*[34] found inverse associations between BMI and serum α-TOH concentrations adjusted for total cholesterol, *i.e.,* the α-TOH:total cholesterol ratio in Mexican-American children of the United States NHANES study. In addition to the association between BMI and α-TOH, BMI has also been positively associated with serum γ-TOH concentrations[32].

It has been observed in several studies that smoking affects serum levels of antioxidants, such as vitamin E. For example, Al-Azemi *et al*[33] and Shah *et al*[38] observed that smokers had lower serum concentrations of α-TOH compared to non-smokers. These findings were supported by Miwa *et al*[39] and Galan *et al*[27] analyzing the female participants of a Japanese study and the cohort of the SU.VI.MAX study, respectively. It is suggested that the dietary patterns of smokers differ from that of non-smokers ultimately leading to differences in vitamin E intake. Interestingly, others have not found differences in vitamin E plasma concentrations between smokers and non-smokers[40,41]. Whereas Mezzetti *et al*[42] observed no differences in plasma vitamin E concentrations between the two groups, vitamin E arterial tissue content was significantly lower in the group of smokers compared to that of non-smokers. This observation may indicate that vitamin E acts as a lipid-soluble antioxidant for the prevention of oxidative damage in the arterial wall. Increased serum concentrations of γ-TOH have also been found both in active and passive smokers by Dietrich *et al*[41]. This finding suggests that excretion of non-α-TOH forms of vitamin E may be decreased to mobilize additional anti-oxidative agents, such as γ-TOH, to increase the body's anti-oxidative capacity. Chronic alcohol consumption also leads to decreased serum levels of α-TOH, partly due to malnutrition[43]. Animal studies have suggested that chronic administration of alcohol results in significantly lower hepatic α-TOH concentrations, probably due to decreased amounts of α-TOH in the mitochondria of hepatocytes[43,44].

INTESTINAL ABSORPTION

When vitamin E is consumed, intestinal absorption is an important factor that limits vitamin E bioavailability. It is known that vitamin E, as a fat-soluble vitamin, follows the intestinal absorption, hepatic metabolism and cellular uptake processes of other lipophilic molecules and lipids[45]. Therefore, intestinal absorption of vitamin E requires the presence of other lipid-rich foods.

In the gastro-intestinal system the absorption rate of vitamin E varies interindividually between 20%-80%[43,45], and is lower than for other fat-soluble vitamins, such as vitamin A[46]. Reasons for individual differences in the absorption rate are diverse. Increased administration of α-TOH with parallel intake of additional food ingredients can decrease the absorption of α-TOH and non-α-TOH forms of vitamin E[47]. For example, retinoic acid[48], plant sterols[49], eicosapentaenoic acid[43], chronic alcohol consumption[43], and dietary fiber[50] are natural food components known to compete with the absorption of vitamin E. How vitamin E is presented to the intestinal surface is crucial for its absorption[19]. In addition, the supplied form of α-TOH, either as a free molecule or, for example, as α-TOH acetate, a common food additive, is of particular importance for its bioavailability[51].

First, triacylglycerols and esterified fat-soluble compounds are partly processed enzymatically in the stomach by gastric lipase[46]. Digestive enzymes including pancreatic lipase, carboxyl esterase and phospholipase A, secreted into the intestinal lumen, continue the digestion of dietary lipids[52]. As vitamin E in the human diet is mostly not esterified, the importance of the lipolytic degradation in the digestion system is likely limited[46], but the need for gastric hydrolysis of the stabilized

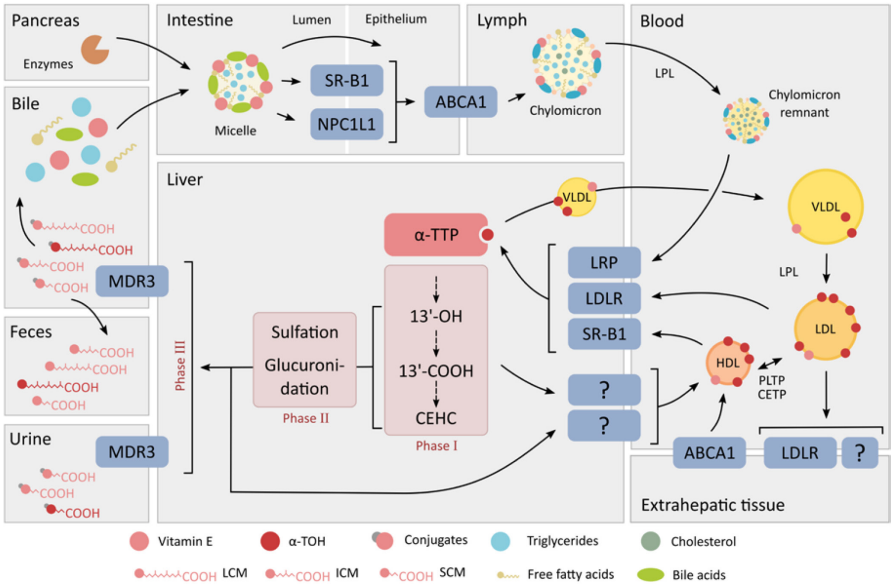

Figure 2 Absorption, transport and metabolism of vitamin E. The route of vitamin E after oral intake follows in general the pathway of other lipids. Pancreatic and intestinal enzymatic digestion followed by the circulation and distribution to the liver and non-hepatic tissues is the same for all vitamin E forms. Discrimination between the different forms of vitamin E in favor of α-TOH occurs mainly in the liver by α-TTP, which protects α-TOH from excessive degradation and excretion. The figure was modified from[43,46,56,60]. SR-B1: Scavenger receptor class B type 1; LPL: Lipoprotein lipase; NPC1L1: Niemann-Pick C1-like 1; VLDL: Very low density lipoproteins; HDL: High density lipoproteins; α-TOH: α-tocopherols; α-TTP: α-TOH transfer protein; LDL: Low density lipoproteins; LRP: LDL receptor-related proteins; LDLR: LDL receptor; 13'-OH: 13'-hydroxychromanol; 13'-COOH: 13'-carboxychromanol; CEHC: Carboxyethylhydroxychromanols; HDL: High density lipoproteins; PLTP: Phospholipid transfer protein; CETP: Cholesteryl ester transfer protein; LCM: Long-chain metabolites; ICM: Intermediate-chain metabolites; SCM: Short-chain metabolites.

forms of vitamin E, such as α-TOH acetate, has not been studied in detail so far[46]. The more stable α-TOH acetate, however, requires further hydrolysis by the bile acid-dependent lipase in the pancreas or an intestinal mucosal esterase[43]. Subsequent absorption of vitamin E in the duodenum is characterized by the transfer from emulsion fat globules to water-soluble multi- and unilamellar vesicles and mixed micelles comprised of phospholipids and bile acids. This is the fundamental step in the gastric digestion and uptake of lipids, and also a crucial phase in the absorption of vitamin E (Figure 2). Since the uptake of vitamin E into enterocytes is less efficient compared to other types of lipids, this may explain the relatively low bioavailability of vitamin E[46]. As mentioned by Desmarchelier *et al*[53], α-TOH acetate is embedded in matrices where its hydrolysis and its uptake by intestinal cells are markedly less efficient than in mixed micelles. The intestinal cellular uptake of vitamin E from mixed micelles follows in principle two different pathways across enterocytes, as has been shown *in vitro* and *in vivo*: (1) passive diffusion; and (2) receptor-mediated transport. Receptors facilitating α-TOH transport across the enterocyte membrane, similar to the intestinal uptake of cholesterol[54], are the class B type 1 scavenger receptor class B type 1 (SR-B1)[55] and the Niemann-Pick C1-like protein 1 (NPC1L1), an apical membrane receptor of the small intestine[56]. The ATP-binding cassette (ABC) transporters ABCG5/ABCG8, at the luminal site, and ABCA1, at the apical site, are responsible for steroid efflux into the intestinal lumen and transport into the lymph system, respectively[57,58]. It has been shown in rats that vitamin E up-regulates these transporters, highlighting the contribution of vitamin E to cholesterol absorption and release[59]. Finally, ABCA1 is directly involved in the export of vitamin E from cells[60]. Presently, an increased intestinal absorption of *RRR*-α-TOH as compared to other stereoisomers[61], or other vitamin E forms such as γ-TOH[62], has not been uncovered. Thereby the efficiency in absorption, and of the integration of α-TOH or γ-TOH into chylomicrons, appears to be comparably equivalent.

VASCULAR TRANSPORT

No specific plasma transport protein for α-TOH has yet been described[43,63]. The transport of vitamin E in blood circulation follows largely that of cholesterol within lipoprotein metabolism[64]. Lipoproteins serve as carriers for lipophilic molecules, such as cholesterol, triglycerides and vitamin E, to distribute them between the liver and different organs and tissues[43]. In principle, the transport of vitamin E is independent of the type of

stereoisomer[65,66]. Under normal physiological conditions, α-TOH is mostly transported *via* chylomicrons, very low density lipoproteins (VLDL) and high density lipoproteins (HDL), whereas under fasting conditions low density lipoproteins (LDL) take on this task[67]. The distribution ratio of α-TOH between the different types of lipoproteins is difficult to define. In a study on fasting normolipemic volunteers, Kostner *et al*[68] found a ratio of 1.0:1.9:1.4 of α-TOH distribution between VLDL, LDL and HDL, respectively. In contrast, Behrens *et al*[69] calculated a ratio of 1.0:9.4:8.4. These variations are most likely due to individual differences in α-TOH distribution in the lipoproteins caused by differing dietary α-TOH intake, metabolic states[68], and other factors influencing bioavailability and the status of vitamin E. In addition, results obtained from human studies regarding the distribution of α-TOH to lipoproteins differ from studies performed on rodents. As shown by Bjørneboe *et al*[67], under non-fasting conditions in rats 51% of serum α-TOH were associated with chylomicrons and VLDL whereas 47% were found within HDL. These differences across species highlight clearly the complexity of this topic.

The concentrations of vitamin E in chylomicrons are independent of the vitamin E form or stereoisomers. Vitamin E is secreted *via* chylomicrons by enterocytes into the intestinal lymph system to begin systemic circulation. The triglycerides in chylomicrons are subjected to hydrolysis by lipoprotein lipase (LPL)[56,70]. During lipolysis, vitamin E remains in the lipoprotein particle and its further transport through circulation occurs *via* chylomicron remnants. Vitamin E is then imported into the liver *via* LDL receptor (LDLR)-related proteins - and LDLR-mediated uptake of chylomicron remnants[60,71].

In the liver, vitamin E undergoes several sorting steps [see section "α-TOH transfer protein (α-TTP)" in the following chapter "intracellular binding proteins"] or metabolic processes (see section "metabolism of vitamin E"). α-Tocopherol is the form of vitamin E that is almost exclusively secreted into circulation *via* VLDL. Whereas VLDL have the highest capacity to carry α-TOH, these particles represent the smallest fraction of lipoproteins involved in vitamin E transport in the circulation in the fasting state[67]. Unfortunately, the mechanisms by which VLDL are enriched with α-TOH are poorly understood[72] (for further details, see the section on α-TTP in "intracellular binding proteins"). Alternatively, analogous mechanisms for α-TOH and free cholesterol have been discussed to explain the intracellular incorporation of α-TOH into nascent VLDL[72]. In support of this hypothesis, Bjornson *et al*[73] found that α-TOH and unesterified cholesterol translocate spontaneously from cellular membranes to lipoproteins. Further, ABCA1 has been shown to enrich HDL with α-TOH, which can be then transfered spontaneously or *via* the plasma phospholipid transfer protein (PLTP) to VLDL[68,74,75]. A similar mechanism has been suggested for the transfer of α-TOH to HDL *via* ABCA1[75].

Similar to chylomicrons, triglycerides in VLDL particles are enzymatically hydrolyzed by LPL resulting in the stepwise formation of LDL. Kono *et al*[76] suggested recently that LDL particles carry the major portion of plasma α-TOH and that LDLR-mediated endocytosis contributes significantly to the uptake of α-TOH into cells[77]. However, studies performed on apolipoprotein B (Apob)-knockout mice and heritable hyperlipidemic Watanabe rabbits lacking the LDLR showed discrepancies in circulating α-TOH levels and tissue distribution, thus questioning the importance of LDL for α-TOH transport[78,79]. Cohn *et al*[79] therefore concluded that α-TOH in LDL can be taken up by tissues *via* LDLR but is also independent of this lipoprotein receptor. Uptake transporters and specific intracellular transport proteins involved in α-TOH trafficking are handled in more detail in a following section.

High-density lipoproteins provides the means for α-TOH to be secreted out of the extrahepatic tissues and enter into circulation to be transported back to the liver. High-density lipoprotein particles contain the lowest concentration of vitamin E per particle but HDL is the most potent donor of vitamin E to several target tissues[64,74,80], despite the larger amounts of LDL in plasma. One possible explanation for this observation was given by Clevidence *et al*[81], who speculated that HDL contain more components for binding α-TOH besides serum ApoAI[69]. The *in vivo* importance of HDL in providing α-TOH to the central nervous system was highlighted by Goti *et al*[80]. In agreement with this finding, Kolleck *et al*[64] found that HDL might be the primary source of vitamin E for type Ⅱ pneumocytes. Furthermore, it has been suggested that the supply of vitamin E by HDL could be more important under pathophysiological conditions, such as oxidative stress, most likely independent from the HDL-mediated uptake of cholesterol[64]. The HDL-interacting scavenger receptor SR-B1 controls the uptake and accumulation of α-TOH in specific tissues[82]. *In vitro* experiments using type Ⅱ pneumocytes identified SR-B1 as a potential α-TOH uptake promoter, whereas the role of SR-B2 in this process is still speculative[64].

Vitamin E or rather α-TOH has been shown to move actively between lipoproteins of different density classes[74]. As previously mentioned, triglycerides in lipoproteins are catabolized *via* LPL[56,70]. During this lipolysis step, vitamin E remains incorporated either in chylomicron remnants (in the case of chylomicrons) or in LDL (in the case of VLDL). PLTP mediates the transfers of α-TOH between different classes of lipoproteins and also the exchange of α-TOH between HDL and cell membranes[68]. Another member of the group of lipid transfer proteins, the cholesteryl ester transfer protein, has also been suggested as having a role in vitamin E transport and metabolism[60] (Figure 2).

Deficiencies due to absorption and vascular transport disorders

As already outlined, Ulatowski and Manor[16] categorized

metabolic deficiencies of vitamin E into primary and secondary deficiencies, where the latter includes disorders of lipid absorption and transport as well as impaired lipoprotein metabolism, such as cholestatic liver disease, short bowl syndrome, Crohn's disease, abetalipoproteinemia and Niemann-Pick disease type C as well as Tangier disease. Depending on the disease, the severity of vitamin E deficiency varies[83]. The causes for fat malabsorption are diverse and usually result in vitamin E deficiency presenting already in early childhood[19]; these include, *inter alia* cholestatic liver diseases, cystic fibrosis[84], Crohn's disease, thrombosis, intestinal pseudoobstruction and chronic pancreatitis. The Marinesco-Sjögren syndrome and chylomicron retention disease, also known as Anderson's disease, are further causes of severe vitamin E deficiency. The symptoms of Anderson's disease are much milder compared to that of the Marinesco-Sjögren syndrome[85,86]. However, both diseases are caused by the inability of enterocytes to assemble or deliver chylomicrons leading to disturbed intestinal fat transport. Abetalipoproteinemia is caused by mutations in the microsomal triglyceride transfer protein that result in impaired intestinal absorption of lipids and severe vitamin E deficiency. In addition, the transport of vitamin E *via* VLDL to extrahepatic tissues is disturbed. Reduced levels of vitamin E are also found in patients with homozygous hypobetalipoproteinemia[83], which is caused by mutations in the *APOB* gene.

Finding a cure for vitamin E deficiency has remained elusive. Administration of α-TOH in micellar form in moderate doses[43] and supplementation of vitamin E are recommended but should be performed without any interfering components, such as some food ingredients. Intramuscular injection or oral uptake as water-soluble vitamin E ester has also been considered[19].

Afamin

As outlined above the vascular transport of vitamin E is performed by lipoproteins. However, in body fluids with low lipoprotein concentrations, such as follicular fluids, an alternative carrier protein for vitamin E, namely afamin, has been described[87-89]. Afamin is a liver-derived plasma glycoprotein and a member of the albumin protein family[90-92]; therefore it is also called α-albumin[93]. Afamin is partly (13%) bound to plasma lipoproteins[88], but circulates mostly in free form. Since afamin has 18 predicted binding sites for vitamin E and shows binding affinity for both α-TOH and γ-TOH, it has been suggested to be an alternative vitamin E transporter in body fluids under conditions where the lipoprotein system is not sufficient for vitamin E transport[89]. Originally, vitamin E was discovered as a resorption-gestations factor in female rats[2,94]. Afamin was therefore suggested as playing a role in female fertility[92], and indeed it has been shown to bind vitamin E and to increase in maternal serum during pregnancy[92]. Yet the precise role of afamin in pregnancy or for infertility is still not known[95]. On the one hand, afamin increases during pregnancy and decreases after childbirth to baseline levels, but on the other hand, a pilot study of Hubalek *et al*[92] showed that women suffering from pregnancy complications had significantly higher median afamin concentrations than women with uncomplicated pregnancy. In addition to being found in follicular fluids, afamin has also been detected in other extravascular fluids, such as cerebrospinal fluids, although the concentration is tenfold lower than in follicular fluids[88]. Since vitamin E deficiency is also known to cause cerebral complications[96], the detection of afamin in cerebrospinal fluids may indicate a role of afamin in neuroprotection[88]. Supporting the suggestion that afamin plays a potential role in fertility and neuroprotection, Jerkovic *et al*[88] found significant correlations of afamin and vitamin E concentrations in follicular and cerebrospinal fluids.

INTRACELLULAR BINDING PROTEINS

α-Tocopherol transfer protein

In contrast to the lipoprotein-mediated transport of vitamin E in the vascular system, cellular vitamin E is specifically bound to intracellular transport proteins, such as α-TTP in the liver where this protein is highly expressed[97]. As α-TTP is also abundantly expressed in the placenta, the importance of α-TOH in preventing fetus resorption is evident[98]. Furthermore, α-TTP is also expressed in several other tissues[76], such as rat brain, spleen, lung and kidney[99], the pregnant mouse uterus[100], retina[101] and central nervous system[21], suggesting an ubiquitous role for α-TTP in intra-organ trafficking[102].

Hosomi *et al*[103] estimated the relative affinities of α-TTP to the different vitamin E forms and stereoisomers starting from *RRR*-α-TOH set to 100%: β-TOH (38%), α-T3 (12%), *SRR*-α-TOH (11%), γ-TOH and trolox (9%) followed by δ-TOH, α-TOH acetate and α-TOH quinone with 2%. Requirements for the binding of vitamin E forms and derivatives to α-TTP are: Three methyl groups at the chromanol ring system (especially at position C5), one free hydroxyl group and the phythyl side-chain[103]. As α-TOH fulfills all of these criteria best within the group of vitamin E forms, it binds efficiently to α-TTP into a deep cavity lined with hydrophobic residues[16], while α-TTP does not readily bind[104] to or transfer γ-TOH[103]. Since the affinity of different vitamin E forms to α-TTP reflects the biological activity from rat resorption-gestations assays[94], the hypothesis is supported that α-TTP is responsible for the discrimination of α-TOH. However, a current study from Grebenstein *et al*[105] raises the suggestion that the metabolism of vitamin E, but not α-TTP, is responsible for discrimination against mainly non-α-TOH forms, as α-TTP protects the side-chain of the different vitamin E forms from ω-hydroxylase-induced degradation. High levels of expression of α-TTP were found to lead to higher intracellular concentrations of γ-TOH in combination with a reduced production of

γ-CEHC, which confirms the concept that binding to α-TTP protects from metabolic degradation[105].

While α-TTP binds both TOHs and T3s, the binding affinity is modulated by the presence of α-TOH, leading to decreased binding of non-α-TOH forms and T3s[106] and, in turn, to improved metabolism of the non-α-TOH forms. Several studies support the observation that α-TOH supplementation depletes plasma and tissue γ-TOH because of the enhanced metabolism of non-α-TOH forms[107,108].

The main function of α-TTP is to maintain normal α-TOH concentrations in the plasma and extrahepatic tissues[72]. This function is ensured by facilitating the transport of α-TOH from the lysosomes to the plasma membranes[109], followed by the continuous export of α-TOH from the liver to the plasma[110]. It is assumed that α-TTP is required for the incorporation of α-TOH into VLDL particles, but the underlying mechanisms are still not understood[72]. Traber *et al*[72] have systematically reviewed the enrichment of VLDL with α-TOH in the ribosomal endoplasmic reticulum, the Golgi apparatus, and the plasma membrane. It is suggested that α-TTP transfers α-TOH into nascent VLDL from the endosome, multi-vesicular bodies and lysosome[72]. Further, Kono and Arai suggested that α-TTP translocates from the cytosol to late endosomes/lysosomes to acquire α-TOH, which has been taken up by endocytosis or released from lipoproteins[76]. The outer membrane of the endocytic vesicles is enriched with both *RRR*-α-TOH and *SRR*-α-TOH by ABCA1, followed by the selective uptake of *RRR*-α-TOH *via* α-TTP[72]. Non-α-TOH forms are not protected against ω-hydroxylation after endocytosis and are further transported to the endoplasmic reticulum and the late endosomal compartment[105]. The α-TTP/α-TOH complex moves to the plasma membrane where it is targeted by phosphatidylinositol bisphosphates (PIP2), which are essential interaction partners of α-TTP[76]. In this case, α-TTP has been shown to transfer α-TOH between membranes through direct protein-membrane interactions[111-113]. In brief, experiments with liposomes have revealed that α-TTP acts as an α-TOH/PIP2 exchanger[76]. A flippase has been suggested to be involved in the transfer of α-TOH to the outer leaflet of the plasma membrane[114]. Next is the spontaneous transfer of α-TOH to nascent VLDL particles in the perisinusoidal space[72]. Interestingly, the effectiveness of the enrichment of nascent VLDL and pre-VLDL with the different stereoisomers *RRR*-α-TOH and *SRR*-α-TOH is similar, although α-TTP is known to be more specific for *RRR*-α-TOH[72]. Hence, the enrichment of VLDL seems to be more complex. However, the Golgi apparatus is probably not involved in this process. As shown by Arita *et al*[115], suppression of the endoplasmic reticulum/Golgi secretory pathway using brefeldin A did not affect the release of α-TOH. These findings suggest that α-TOH can be secreted by the liver independently from VLDL, although post-secretory nascent VLDL has not yet been eliminated as a physiological α-TOH acceptor[72].

Familial isolated vitamin E deficiency, which is also known as ataxia with isolated vitamin E deficiency (AVED), is categorized as a primary vitamin E deficiency. Symptoms of AVED are ataxia, dysarthria, reduced or absent tendon jerks, and impaired vibration sense. AVED is an autosomal recessive disorder caused by mutations in the *TTPA* gene[76], which result in the inadequate distribution of vitamin E to peripheral tissues[116,117]. Many of the 20 different mutations known to underlie AVED encode for truncated and therefore defective α-TTP proteins[76]. While absorption and vascular transport of vitamin E is normal in these patients, the release of α-TOH from the liver into circulation is disturbed[118]. Due to the dysfunctional α-TTP, AVED patients show impaired selectivity between α- and γ-TOH[119] and no differentiation between *RRR*- and *SRR*-α-TOH[72]. As a consequence of the lack of α-TTP, lower α-TOH plasma concentrations are found in AVED patients, likely due to increased rates of metabolic degradation of α-TOH, measureable as significantly increased urinary excretion of α-CEHC, the end-product of vitamin E catabolism, compared to healthy individuals[116]. It was therefore concluded that the capacity of α-TTP rather than the plasma concentration of α-TOH regulates the rate of vitamin E degradation[120].

Kono *et al*[76] found in mice with different genetic α-TTP backgrounds, with respect to the *Ttpa* gene encoding α-TTP, such as *Ttpa*$^{+/+}$, *Ttpa*$^{+/-}$ and *Ttpa*$^{-/-}$, that α-TOH plasma levels correlate with the number of functional *Ttpa* alleles, which supports the aforementioned conclusion. Similarly to human AVED patients, *Ttpa* knockout mice are "characterized by vitamin E deficiency, oxidative stress, late-onset ataxia, and female infertility, all of which can be prevented by timely supplementation with α-tocopherol"[16]; the *Ttpa* knockout mouse seems to be therefore a suitable animal model to study the role of α-TTP in vitamin E homeostasis.

Other binding proteins

In addition to α-TTP, further intracellular α-TOH binding and transport proteins are known, namely the tocopherol-associated protein (TAP) and the tocopherol-binding protein. In humans, three highly homologous TAP proteins, namely TAP1/SEC14-like 2 protein (SEC14L2; synonymously, supernatant protein factor, SPF), TAP2/SEC14L3 and TAP3/SEC14L4, have been described[121,122], but the contribution of TAP2 and TAP3 to the transport and metabolism of vitamin E has not yet been investigated. TAP 1 and α-TTP belong to a family of ligand-binding proteins that have a *cis*-retinal binding motif sequence, the so-called CRAL-TRIO domain. All members of this protein family bind α-TOH, but to a lesser extent than α-TTP, and the physiological relevance is unknown[19]. It has been suggested that TAP1 is involved in intracellular trafficking of α-TOH[56,123]; TAP1 mediates anti-proliferative effects in LNCaP and DU-145 prostate cancer cell lines by promoting vitamin E uptake

but also by effects independent of α-TOH[1,124]. TAP 1 is involved in cholesterol synthesis and forms complexes with *RRR*-α-TOH quinone, the oxidation product of α-TOH; however, for a better understanding of the function of TAP1, further studies are needed[19,125].

In addition to the binding and transport proteins with affinity and specificity for α-TOH, the cytosolic protein saposin B has a specific binding site for γ-TOH[126]. Saposin B binds γ-TOH more effectively than α-TOH when these vitamin E forms are competing for binding to saposin B *in vitro*[126]. However, *in vivo* the concentration of α-TOH is tenfold higher than of γ-TOH, leading to widely differing conditions which characterize the *in vitro* binding affinity studies in which a ratio of γ-TOH to α-TOH of 1:5 was used.

METABOLISM OF VITAMIN E

Simon products

Many decades ago it was thought that the metabolism of vitamin E was initiated by a radical attack on the chroman structure resulting in a ring opening and the building thereby of TOH quinone[127]. Subsequent side-chain degradation would lead to α-tocopheronic acid and its lactone, α-tocopheronelactone (α-TL), the so-called Simon metabolites described in conjugated and non-conjugated forms in urine of mice and humans[120,128-130]. This pathway has since been questioned because: (1) urinary TOH metabolites with an intact chroman ring system, the carboxyethylhydroxychromanols (CEHC), have been discovered[131-133]; and (2) an almost complete conversion of α-CEHC to α-TL is possible at least *in vitro* by bubbling oxygen through a solution of 70 mmol/L α-CEHC in 0.1 mol/L HCl for 24 h at room temperature[132,134]. In contrast, a recent study by Sharma *et al*[135] provided evidence that conjugates of α-TL are indeed real metabolites and not methodological artefacts. In addition, the study characterized α-TL as a biomarker of oxidative stress in children with type 1 diabetes, as the mean concentrations of the glucuronides and sulfate conjugates of α-TL were all significantly increased in these children[135]. Further research is required to answer the question of whether α-TL is a definite marker or an analytical artefact.

Hepatic metabolism of vitamin E

Degradation processes in the hepatic metabolism of vitamin E remain poorly understood. Initial mechanisms are generally accepted, *i.e.,* all vitamers are degraded to vitamer-specific physiological metabolites with an intact chromanol ring (therefore the nomenclature as α-, β-, γ- and δ-metabolites is used), leading to changes in the side-chain. The metabolites have been found in different tissues and body fluids conjugated similarly to xenobiotics and in non-conjugated form. For a detailed overview, see Figure 3.

Metabolism of TOH

Hepatic metabolism of TOH is initiated by CYP4F2/CYP3A4 (for details, see the sections on CYP3A4 and CYP4F2 in chapter "metabolism of vitamin E") dependent ω-hydroxylation of the aliphatic side-chain, forming 13′-hydroxychromanol (13′-OH), which can be analyzed using GC-MS (γ-, δ-13′-OH[139,140]). Next, ω-oxidation leads to 13′-carboxychromanol (13′-COOH), which can be detected *via* LC-ESI-MS (γ-, δ-13′-COOH[139,136]), HPLC-FD or HPLC-ECD (γ-, δ-13′-COOH[136,141]) or GC-MS (γ-13′-COOH[140]). An overview of all metabolites of TOH, analytical methods and identified matrices is provided in Table 1. Subsequent β-oxidation steps shorten the side-chain, thus forming carboxydimethyldecylhydroxychromanol (CDMDHC, 11′-COOH) followed by carboxymethyloctyl-hydroxychromanol (CDMOHC, 9′-COOH), both of which can be analyzed by LC-ESI-MS (γ-, δ-11′-COOH and γ-, δ-9′-COOH[139,136,141]), HPLC-FD or HPLC-ECD (γ-, δ-11′-COOH and γ-, δ-9′-COOH[136,141]) or GC-MS (γ-11′-COOH and γ-9′-COOH[140]). These metabolites with a side-chain length of between 13 to 9 carbon units can be summarized as long-chain metabolites (LCM) of vitamin E. According to their hydrophobicity, the LCMs are not excreted into urine and have been found in human and rat liver microsomes (α-, γ-13′-OH, and α-, γ-13′-COOH[139,140]), murine serum (γ-, δ-11′-COOH[136]), as well as human serum (α-13′-COOH[142]), in human and mice feces (α-13′-COOH; γ-, δ-11′-COOH; γ-, δ-9′-COOH[136]), and *in vitro* in cell culture supernatants of human lung epithelial A549 cells (γ-, δ-13′-OH and γ-, δ-13′-COOH[139]), as well as in HepG2 cells (γ-, δ-13′-OH; γ-, δ-13′-COOH; γ-11′-COOH; γ-9′-COOH[140,143]).

Intermediate-chain metabolites (ICM) are the products of two further β-oxidation steps. Carboxymethylhexylhydroxychromanol (CDMHHC, 7′-COOH) and carboxymethylbutylhydroxychromanol (CMBHC, 5′-COOH) have been detected *via* GC-MS (γ-7′-COOH and α-, γ-5′-COOH[134,140,144,137]), HPLC-ECD (γ-, δ-7′-COOH and α-, γ-, δ-5′-COOH[136,137]), and LC-ESI-MS (γ-, δ-7′-COOH and α-, γ-, δ-5′-COOH[136]) in murine liver (γ-, δ-5′-COOH[136]), in murine serum (γ-, δ-5′-COOH[136]), as well as in human serum or plasma (α-, γ-5′-COOH[136,116]), in murine and human feces (γ-, δ-7′-COOH; α-, γ-, δ-5′-COOH[136]), and in murine urine (γ-, δ-5′-COOH[136]), as well as in human urine (α-, γ-, δ-5′-COOH[134,136,144]). The ICMs have also been found in cultured HepG2 cells (α-, γ-7′-COOH and α-, γ-5′-COOH[120,140,144]).

The catabolic end-products of vitamin E metabolism are the CEHC (CEHC, sometimes also referred to 3′-COOH or short-chain metabolites, SCM), which were an early focus of research on vitamin E metabolism[145,146]. In the 1980s and 1990s different CEHCs were identified as the first known metabolites of vitamin E degradation: α-CEHC[132,146], γ-CEHC[147] and δ-CEHC[131]. Shortly after the discovery of the SCMs, it was shown that not only γ-TOH metabolism but also the degradation of γ-T3 results in γ-CEHC[148]. Meanwhile, this has been confirmed for all T3s and their corresponding CEHCs[136]. Many different analytical procedures and detection

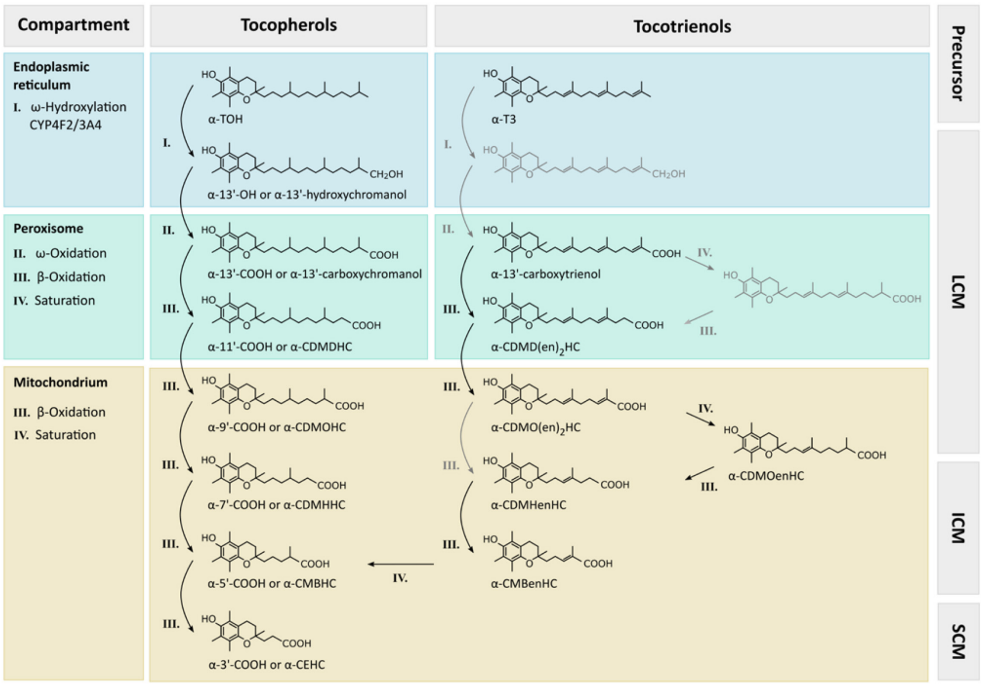

Figure 3 Metabolism of vitamin E. The metabolism of vitamin E starts with one cycle of ω-hydroxylation followed by five cycles of β-oxidation. The principal catabolic pathway is independent of the saturation of the side-chain[136,137] or the substitution of the chromanol ring system[136], whereas the rate of metabolic degradation is modulated mostly by these two factors[136] (for further details, see the sections on α-TTP in chapter "Intracellular binding proteins" as well as CYP4F2 and kinetics in chapter "Metabolism of vitamin E"). While the intracellular compartmentation of the different reaction steps is known for TOHs[138], it remains unresolved for T3s. The same applies to the structures and arrows marked in grey; they reflect molecules or principle steps which fit into the general concept so far but need further experimental confirmation and molecular characterization. α-TOH: α-tocopherols; α-T3: α-tocotrienols; 13'-OH: 13'-hydroxychromanol; 13'-COOH: 13'-carboxychromanol; LCM: Long-chain metabolites; ICM: Intermediate-chain metabolites; SCM: Short-chain metabolites; CDMD(en)2HC: Carboxydimethyldecadienylhydroxychromanol; CDMOenHC: Carbodimethyloctenylhydroxychromanol; CDMHenHC: Carboxymethylhexenylhydroxychromanol; CMBenHC: Carboxymethylbutadienylhydroxychromanol; CDMOHC: Carboxymethyloctylhydroxychromanol; CDMHHC: Carboxymethylhexylhydroxychromanol; CMBHC: Carboxymethylbutylhydroxychromanol; CEHC: Carboxyethylhydroxychromanols.

methods for the SCMs have been described: LC-ESI-MS (α-, γ-, δ-CEHC[139,136,141]), HPLC-ECD (α-, γ-CEHC[136,137,149-152]), and GC-MS (α-, γ-, δ-CEHC[134,144,137,153]). Usually, trolox or 1-naphthol are used as internal standards for the analysis of SCMs[150,151,154]. The CEHCs have been analyzed in rat and murine liver (α-, γ-, δ-CEHC[136,154]), in rat plasma and in mice serum (γ-, δ-CEHC[136,141]), as well as in human plasma and serum (α-, γ-, δ-CEHC[140,136,150,152,155]), in human and mouse urine (α-, γ-, δ-CEHC[134-136,144,151,155,156]) and in human and mouse feces (α-, γ-, δ-CEHC[136]) as well as in rat bile (α-, γ-CEHC[149]) and *in vitro* in A549 and HepG2 cells (γ-CEHC[140,141,144,137]).

Recently, two new metabolites, namely 12′-hydroxychromanol (12′-OH: γ- and δ-12′-OH) and 11′-hydroxychromanol (11′-OH: γ- and δ-11′-OH) have been identified using GC-MS in fecal pellets of mice fed a diet rich in γ-TOH[157]. These products provide evidence for ω-1 and ω-2 hydroxylation activity and that 12′-OH is not able to undergo oxidation followed by side-chain truncation. These metabolites are therefore excreted *via* bile and are found in the feces of mice and humans[157].

Metabolism of T3

The metabolism of T3s was first analyzed by Birringer *et al*[137] in HepG2 cells; it is in principal comparable to the metabolism of TOHs starting with an ω-hydroxylation followed by five cycles of β-oxidation and resulting in the end-product CEHC. Meanwhile almost all expected metabolites have been found in mouse and human samples after supplementation with their respective metabolic precursors, TOHs and T3s[136]. The corresponding metabolites were found: Carboxytrienol (13′), carboxydimethyldecadienylhydroxychromanol [CDMD(en)$_2$HC; 11′], carbodimethyloctenylhydroxychromanol (CDMOenHC; 9′), as well as carboxydimethyloctadienylhydroxychromanols [CDMO(en)$_2$HC; 9′], carboxymethylhexenylhydroxychromanol (CMHenHC; 7′), and carboxymethylbutadienylhydroxychromanol (CMBenHC; 5′). This leads to the conclusion that the side-chain of T3s is saturated before shortening. We

Table 1 Overview on metabolites of vitamin E

Metabolite	Methods	Matrix
13′-Hydroxychromanol (13′-OH)	GC-MS[139,140]	Human and rat liver microsomes[139,140] Cell culture medium of A549 cells[139] HepG2 cells[140,143]
13′-Carboxychromanol (13′-COOH)	LC-ESI-MS[136,139] HPLC-FD or HPLC-ECD[136,141] GC-MS[140]	Human liver microsomes, serum, feces[136,140,142] Rat liver microsomes[139,140] Mouse feces[136] Cell culture medium of A549 cells[139] HepG2 cells[140,143]
Carboxydimethyldecyl-hydroxychroman (CDMDHC) (11′-COOH)	LC-ESI-MS[136,139,141] HPLC-FD or HPLC-ECD[136,141] GC-MS[140]	Human feces[136] Mouse serum and feces[136] HepG2 cells[140,143]
Carboxymethyloctyl-hydroxychroman (CDMOHC) (9′-COOH)	LC-ESI-MS[136,139,141] HPLC-FD or HPLC-ECD[136,141] GC-MS[140]	Human and mouse feces[136] HepG2 cells[140,143]
Carboxymethylhexyl-hydroxychroman (CDMHHC) (7′-COOH)	GC-MS[134,137,140,144] HPLC-ECD[136,137] LC-ESI-MS[136]	Human and mouse feces[136] HepG2 cells[120,140,144]
Carboxymethylbutyl-hydroxychroman (CMBHC) (5′-COOH)	GC-MS[134,137,140,144] HPLC-ECD[136,137] LC-ESI-MS[136]	Human serum or plasma, urine and feces[116,134,136,144] Mouse liver, serum, urine and feces[136] HepG2 cells[120,140,144]
Carboxyethyl-hydroxychroman (CEHC) (3′-COOH)	LC-ESI-MS[136,139,141] HPLC-ECD[136,137,149-152] GC-MS[134,137,144,153]	Human plasma or serum, urine, feces[134-136,140,144,150-152,155,156] Rat liver, plasma, bile and urine[141,149,154] Mouse serum or plasma, urine, feces and liver[134-136,144,151,155,156] A549 and HepG2 cells[137,140,141,144]

speculate that auxiliary enzymes also needed for the degradation of unsaturated fatty acids, *e.g.*, 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase, are therefore required for the saturation of the double bonds in the side chain of T3 metabolites, as was originally suggested by Birringer *et al*[137].

Intracellular compartmentation of vitamin E metabolism

Mustacich *et al*[138] were the first to identify the intracellular compartmentation of vitamin E metabolism. Based on their work, the ω-hydroxylation catalyzed by alcohol dehydrogenase (resulting in 13′-OH) and the following reaction catalyzed by aldehyde dehydrogenase (forming 13′-COOH), take place at the endoplasmic reticulum. The carboxylated side-chain of TOHs are similar in structure to 2-methyl branched-chain fatty acids, which are subsequently β-oxidized in peroxisomes by their activation to an acyl-CoA ester[138]. This pattern seems to also characterize degradation of LCMs as two cycles of peroxisomal β-oxidation (resulting in 11′-COOH and 9′-COOH) with separation of propionyl-CoA or acetyl-CoA were suggested for TOHs[138]. Cho *et al*[156] noted that the sterol carrier protein-x (SCP-x) is involved in peroxisomal oxidation of branched-chain lipids, serves as a peroxisomal 3-ketoacyl-CoA thiolase, and shows reduced expression in response to pregnane X receptor (PXR) activation (for further details on PXR and vitamin E interactions, see the section on CYP3A4 in chapter "metabolism of vitamin E"). Based on their data the authors suggested that SCP-x is involved in the formation of vitamin E metabolites. It is not known yet whether LCMs are transported into peroxisomes as "free"molecules or as CoA esters, as it is known for long-chain, very long-chain or branched-chain fatty acids[158]. Based on knowledge of the transport of very long-chain acyl-CoA into the peroxisomes *via* ABCD[159], an involvement of related proteins for the import of vitamin E metabolites could be possible. As the last degradation steps are located in the mitochondria[138], the transport mechanisms out of peroxisomes and the subsequent import into mitochondria also needs to be unraveled. The import into mitochondria might occur through carnithin-acyl-transferases, as reported for metabolites of vitamin K[160]. Grebenstein *et al*[105] suggested that the LCMs are possible ligands for α-TTP and/or other hepatic TOH binding proteins. However, the importance of these proteins for the transfer of LCMs to mitochondria needs to be investigated further[105]. The mitochondrial production of ICMs and SCMs (three β-oxidation steps) is indicated by the analysis of CEHCs solely in mitochondria but not in peroxisomes[138]. It was emphasized by Mustacich *et al*[138] that these data do not exclude a model of exclusive mitochondrial β-oxidation.

Enzymatic degradation of vitamin E

As mentioned above, catabolism of TOHs and T3s begins with an ω-hydroxylation of the side-chain which is catalyzed by cytochrome P450 (CYP) enzymes, namely CYP4F2 or CYP3A4. This oxidation is the rate-limiting step in vitamin E metabolism[161]. CYP enzymes are heme-thiolate proteins that differ in substrate selectivity but catalyse monooxygenation reactions *via* activation of molecular oxygen[162]. In the following sections, we provide an overview of the two CYP enzymes known to be involved in vitamin E metabolism.

CYP3A4: The enzyme CYP3A4 is the most important CYP enzyme in humans, as the majority of administrated drugs are metabolized *via* CYP3A4 due to its wide range of substrates[162]. The first evidence for the involvement of CYP3A4 in vitamin E metabolism was provided by Parker *et al*[163]. Ketoconazole, an inhibitor of CYP3A4, blocked the metabolism of TOHs [α- and γ-TOH in primary rat hepatocytes or γ- and δ-TOH in HepG2/C3A (25 μmol/L each)] to their corresponding SCMs by approximately 90% after incubation with 1 mmol/L or 0.25 mmol/L for 48 h[163]. Similar effects were seen for γ-TOH when 1 mmol/L sesamin was used, a major sesame seed lignin and natural inhibitor of CYP3A4[163]. When 50 mg/kg body weight of ketoconazole were applied by oral gavage to rats simultaneously with a mixture of 10 mg/kg body weight of α-TOH, 10 mg/kg body weight of γ-TOH or 29.5 mg/kg body weight of T3 the catabolism of the vitamin E forms to their respective SCMs and excretion *via* urine was clearly reduced compared to the controls[164]. Using 50 μmol/L rifampicin, an inducer of CYP3A4 activity, Birringer *et al*[120] demonstrated an up to fivefold increase in *all-rac*-α-TOH degradation in HepG2 cells. It should be noted that preconditioning of the cells with 100 μmol/L α-TOH for 10 d was necessary, as the standard cell culture medium is deficient for α-TOH[120]. This might be the reason why Parker *et al*[163] were not able to detect α-TOH metabolism in HepG2 cells, as they did not perform a preconditioning and instead incubated the cells only for 24 h to 48 h with 0.25 μmol/L or 0.5 μmol/L α-TOH. Further evidence is given by the α-TOH-dependent regulation of CYP3A4[165]. Feeding mice with 200 mg/d of α-TOH for nine months resulted in 1.7-fold higher Cyp3a11 (*i.e.,* the murine orthologue of human CYP3A4) mRNA expression levels compared to after three months, while γ-T3 did not increase Cyp3a11 mRNA levels[166]. Similar effects on Cyp3a protein levels were observed when 10 mg/100 g body weight of α-TOH was injected subcutaneously into rats[167]. Traber *et al*[165] showed that C57BL/6 mice receiving a diet sufficiently enriched with α-TOH (31 mg α-TOH per kg diet) for five weeks have increased hepatic Cyp3a levels compared to mice fed an α-TOH-deficient diet with less than < 2 mg α-TOH per kg diet.

Several CYP enzymes, including CYP3A4, are regulated by structurally diverse xenobiotics *via* PXR, a nuclear receptor that regulates the expression of metabolic enzymes and transporters involved in the metabolism of xenobiotics and endobiotics[156,168]. Landes *et al*[169] showed that vitamin E acts as an agonist of PXR. Thereby, the inductive effect of vitamin E on chloramphenicol acetyl transferase activity was dependent on the vitamin E form in the following order: γ-T3 approximately equal α-T3 > rifampicin > δ-TOH > *RRR*-α-TOH ≥ γ-TOH. The treatment of HepG2 cells with γ-T3 led to an up-regulation of CYP3A4 and CYP3A5 mRNA levels[169] and a dose-dependent activation of chloramphenicol acetyl transferase at 1 μmol/L to 10 μmol/L, concentrations which can be reached also in human plasma[23]. These striking contrary effects of γ-T3, up-regulation of CYP3A4 expression *in vitro*[168] and no effect[166] or even a reduction of Cyp3a expression[165] *in vivo* might be explained by different availabilities of the individual vitamin E forms at the site of action. Whereas the substance *in vitro* is applied onto the cells directly, all physiological processes of vitamin E handling, especially α-TTP dependent sorting of non-α-TOH forms in combination with the high metabolic degradation and elimination rates for γ-T3[166], may interfere *in vivo*, thus resulting in contradictory effects.

However, the induction of Cyp3a11, the mouse homologue of human CYP3A4, by α-TOH and the involvement of PXR was confirmed in wild-type mice by Johnson *et al*[170]. In this study, mice kept vitamin E-deficient were fed daily for two weeks with 500 mg/kg *DL*-α-TOH acetate, which is equivalent to a typical supplementation with 400 - 600 mg/d α-TOH for a 70 kg human. Expression of Cyp3a11 was induced in wild-type mice, but remained unchanged in Pxr-null mice and in humanized PXR mice as well, while dosing with known murine and human PXR-specific agonists up-regulated expression of Cyp3a11 in both the wild-type and the humanized PXR mice, but not the Pxr-deficient mice. This led to the conclusion that α-TOH is a partial agonist of mouse Pxr and that Pxr is required for the induction of Cyp3a11 by α-TOH in mice[170].

Apart from these, there are further contradictory findings. Parker *et al*[171] emphasized that the hypothesis of involvement of CYP3A4 in the metabolism of vitamin E is only based on the assumption of ketoconazole specificity, which proved incorrect[172]. Testing recombinant human CYP3A4 in insect cell derived microsomes revealed no activity towards α- or γ-TOH[140], whereas a systematic screening of other CYP enzymes showed tocopherol-ω-hydroxylase activity only for CYP4F2[171] (for further details, see the section on CYP4F2 in chapter "metabolism of vitamin E"). In addition, Birringer *et al*[120] showed that production of γ-CEHC from γ-TOH was not affected by rifampicin in HepG2 cells, leading to the conclusion that either CYP3A4 may not degrade all vitamin E forms to the same extent or other CYP enzymes may be involved in γ-TOH metabolism. Furthermore, Schuetz *et al*[173] reported in 1993 that HepG2 cells do not express CYP3A4 but CYP3A7. With respect to PXR, more discrepancies have been reported. Cho *et al*[156] used *Pxr*-deficient *vs* wild-type mice both treated with and without pregnenolone 16α-carbonitrile (an activator of rodent Pxr) to analyze the impact of Pxr on vitamin E degradation. The study revealed that urinary excretion of α-CEHC glucuronide was significantly decreased down to 16% and γ-CEHC glucoside was attenuated down to 40% in pregnenolone 16α-carbonitrile-treated wild-type mice compared with control wild-type mice, while urinary excretion of both metabolites were unaffected in the *Pxr*-null mice. Johnson *et al*[170] suggested that these findings

Table 2 Experimentally validated conjugates of vitamin E metabolites

Metabolite	Conjugate	Organism
LCM	Sulfates	Rats and *in vitro*[139,141]
ICM	Ether glucuronides	Human and mouse urine[176]
	Sulfates	Human urine[177]
SCM	Glucosides, glutamine	Mouse[156,176]
	Taurine, glycine or glycine glucuronides	Human and mouse urine[176]
	Sulfates	Rats[178]

LCM: Long-chain metabolites; ICM: Intermediate-chain metabolites; SCM: Short-chain metabolites.

are the result of a down-regulation of β-oxidation by pregnenolone 16α-carbonitrile. To sum up, the role of CYP3A4 in the metabolism of vitamin E remains unclear.

CYP4F2: The CYP4 subfamily of CYP enzymes catalyzes the ω-hydroxylation of saturated, branched-chain fatty acids as well as unsaturated fatty acids, whereas members of the CYP4F subfamily metabolize long-chain and very long-chain fatty acids[158]. As mentioned above, Sontag and Parker[140] reported the involvement of CYP4F2 in vitamin E metabolism using reporter-gene assays combined with a systematical screening of CYP enzymes in this context. Among the CYP enzymes tested, only CYP4F2 exhibited tocopherol-ω-hydroxylase activity, which was higher for γ-TOH than for α-TOH. In a subsequent study, Sontag and Parker[108] characterized the substrate specificity of CYP4F2. According to this study, the unsubstituted carbon at position C5 of the chromanol ring system induces activity of CYP4F2, so that γ- and δ-TOH are metabolized more efficiently than α-TOH, which in turn stimulates the metabolism of other vitamin E forms. The authors found higher V_{max} values for T3s than for their corresponding TOHs, suggesting that CYP4F2 contributes to the preferential physiological retention of α-TOH compared to other vitamin E forms. This finding supports the central role of this pathway in modulating the vitamin E biopotencies of TOHs and T3s[140,108]. Bardowell *et al*[161] identified Cyp4f14 as the murine orthologue of human CYP4F2 and analyzed vitamin E homeostasis in Cyp4f14 knock-out mice. Cyp4f14-deficient mice had higher tissue concentrations of non-α-TOH forms, such as γ-TOH in plasma and tissues and δ-TOH in fat tissue, whereas tissue and plasma levels of α-TOH remained unchanged (except for lower concentrations in heart tissue). In line with these findings, reduced elimination of γ-, δ-, and α-TOH metabolites *via* urine and feces was found, but increased fecal excretion of γ- and δ-TOH[161]. Due to the reduction of vitamin E metabolism instead of a complete abolishment, Bardowell *et al*[161] suggested the involvement of other enzymes in murine vitamin E catabolism.

While expression of CYP3A4 is regulated by α-TOH, CYP4F2 levels are not influenced by α-TOH as reported by Mustacich *et al*[167] in rats that were injected subcutaneously daily with 10 mg/100 g body weight α-TOH for up to 18 d. In these rats, protein levels of Cyp3a, Cyp2b, and Cyp2c were increased. Johnson *et al*[170] found that only the mouse orthologue of CYP4F2, Cyp4f13, but not Cyp4f14 was upregulated by α-TOH in wild-type mice, while in *Pxr*-null or humanized PXR mice no influence on the expression of Cyp4f13 and Cyp4f14 was observed. In *Ttpa*$^{-/-}$ and wild-type mice, Traber *et al*[165] revealed no influence of γ-TOH on the expression of the Cyp4f13 protein. However, a synthetic inhibitor of CYP4F2/Cyp4f13, namely (R)-2-[9-(1H-imidazol-1-yl)nonyl]-2,5,7,8-tetramethylchroman-6-ol, decreased formation of γ-CEHC from γ-TOH in HepG2 cells in culture[174]. The stable expression of CYP4F2 *in vivo* regardless of elevated vitamin E intake or availability, as indicated by higher concentrations of the metabolites, calls into question the suggestion that CYP4F2 alone is responsible for the initial step of vitamin E degradation. Taken together, the evidence for the involvement of CYP4F2 in vitamin E metabolism is convincing, but the participation of other CYP enzymes such as CYP3A4 cannot be excluded yet.

Conjugation of metabolites: Non-α-TOH forms of vitamin E are preferentially handled in the human body as xenobiotics involving phase I enzymes, and further degradation steps seem to also follow the track of xenobiotic metabolism, as vitamin E degradation products are found as sulfate and glucuronide conjugates. See Table 2 for an overview on the conjugates of vitamin E metabolites identified thus far.

Sulfates are thought to be the main conjugation products of the LCMs (γ- and δ-LCMs: 13′-COOH, 11′-COOH and 9′-COOH in rats and *in vitro*)[139,141], as an unknown peak occurring only after supplementation with γ-TOH in rats had the theoretical weight of sulfated γ-CEHC. In agreement with this finding, the peak decreased after treatment with sulfatase[139] or a combination of β-glucuronidase and sulfatase[141]. As the LCMs were found in both conjugated and non-conjugated form in cell culture medium of human A549 cells, it was suggested that conjugation and subsequent β-oxidation are parallel processes. This was confirmed in studies by Hashiguchi *et al*[175]. α-5′-COOH ether glucuronides[176] in urine of both humans and mice as well as α-5′-COOH sulfate in human urine[177] are known conjugates of ICMs. More is known about the conjugates of the SCMs. In humans, the majority of CEHCs are excreted *via* urine as glucuronides[136,147,152,155], whereas sulfates were found only in trace amounts[177] or in significant amounts[135,136,176]. In addition, other conjugates are known for α-CEHC, such as taurine, glycine or glycine glucuronide conjugates[176]. In mice, glucuronides and sulfates of α-, γ-, δ-CEHC have been found[136,156,176]. While glucoside conjugates of γ-CEHC[156] and α-CEHC glutamine[176] have been only found in mice, taurine, glycine or glycine glucuronides conjugates were found

in murine urine as well as in urine of humans[176]. The glucoside conjugate of γ-CEHC was found by Cho *et al*[156] and appeared to be the main conjugated form of γ-CEHC in mice. A comparative experiment revealed that β-glucuronidase treatment hydrolyzed not only glucuronide conjugates but also glucose-conjugated metabolites; it has been therefore suggested that glucosides originally contained in the samples were not detectable when β-glucuronidase treatment was applied[156]. As this procedure is common in vitamin E metabolite analysis, it is possible that glucosides remained mostly undetected. Tanabe *et al*[178] found sulfated γ-CEHC to be the main excretion product in rats, when γ-CEHC was applied; a conjugation product with the expected weight of sulfated γ-CEHC was detected and was also sensitive to β-glucuronidase/sulfatase treatment. This finding was confirmed by others, when sulfated γ-CEHC in rat urine or plasma was found[139,141,179].

To address the ratio of conjugated to non-conjugated metabolites in different body fluids, parallel analyses with or without enzymatic hydrolysis were used. Most CEHCs in urine are conjugated, as several groups have reported. Zhao *et al*[136] found a six- to tenfold increase of free CEHC in mouse urine after treatment with β-glucuronidase and sulfatase. Freiser and Jiang[141] suggested that between 88% to 98% of γ-CEHC is conjugated in the plasma of rats, and Leonard *et al*[154] found that between 30% to 40% of the tested α- and γ-CEHCs are conjugated in the liver. Lodge *et al*[151] suggested analysis of the portion of non-conjugated SCMs, as γ-CEHC was shown to be a powerful natriuretic factor in human urine[133].

The conjugates of vitamin E metabolites identified so far indicate an involvement of phase Ⅱ enzymes, mainly UDP glucuronosyltransferases (UGT) and sulfotransferases (SULT). UDP glucuronosyltransferase enzymes are involved in the conjugation of CEHCs with glucuronic acid or glucose[156], whereas SULT catalyze the transformation to sulfate conjugates. Interestingly, the different vitamin E forms have no effect on the expression of phase Ⅱ enzymes, as *in vitro* none of the eight vitamin E forms showed an altered expression of UGT1A1 mRNA in human primary hepatocytes[180]. Furthermore, the majority of UGT isoforms were not regulated in mice fed α-TOH deficient or enriched diets[170], and hepatic UGT activity was not influenced by feeding rats daily with 200 mg/kg body weight α-TOH for two weeks. It is of note that expression of UGT1A1 mRNA was increased 1.7-fold after treatment of wild-type mice with the PXR agonist pregnenolone 16α-carbonitrile compared to untreated control mice[156]. As UGTs are regulated *via* PXR[181,182], the effect of pregnenolone 16α-carbonitrile is in line with the latter finding, while the lack of UGT activation by α-TOH was unexpected. In a comparative screening of 14 members of the SULT enzyme family, Hashiguchi *et al*[175] found evidence for the involvement of SULT1 enzymes in vitamin E metabolism. Sulfotransferase 1 showed a stronger preference for γ-TOH than for α-TOH and for γ-CEHC over all other CEHCs. Contradictory results were obtained in mice where expression of Sult2a mRNA was increased 10.8-fold by α-TOH compared to the control mice[183]. Mustacich *et al*[138] noted that the presence of sulfated metabolites is difficult to clarify with respect to the exclusive peroxisomal degradation of vitamin E and the cytosolic localization of phase Ⅱ enzymes. However, when metabolites are generated in peroxisomes and mitochondria, they must be transported through the cytoplasm and can be subjected as substrates to the cytosolic SULTs[138]. Jiang *et al*[139] noted that sulfated metabolites of vitamin E may not only contribute to detoxification but could also perform regulatory functions. As mentioned before, in humans and mice, the concentration of glucuronides is higher than that of sulfates and a ratio of glucuronide:sulfate of approximately 8 was reported by Sharma *et al*[135]. The authors argued that this is in line with the fact that humans have higher capacity for glucuronidation than sulfation because of the high activity of hepatic UGT.

Another enzyme involved in the xenobiotic metabolism is glutathione S-transferase (GST). Van Haaften *et al*[184,185] found that TOHs and T3s inhibit isolated human GST P1-1 with IC_{50} values of 0.7 mmol/L for α-TOH, 0.8 mmol/L for δ-TOH, 1.8 mmol/L for α-T3 and 0.7 mmol/L for γ-T3. Contradictory findings were reported by Podszun and Frank[186] in rats fed high doses of α-TOH (2500 mg/kg diet for ten days), in which GST activity was increased 2- to 3-fold. When mice were fed with 1000 mg/kg diet of *all rac*-α-TOH acetate for four months expression of hepatic Gstm3 mRNA, responsible for the detoxification of electrophilic compounds, increased about twofold compared to the controls (35 mg *all rac*-α-TOH acetate/kg diet)[183].

Excretion: Due to their polarity, ICMs and SCMs, namely 5'-COOH and CEHCs, are excreted *via* urine, mostly as glucoside conjugates[136]. Feces contain the whole set of vitamin E metabolites, including precursors (TOHs and T3s) and water-soluble SCMs in humans[136] and mice[157,161,187]; LCMs (especially 13'-COOH) are the main fecal metabolites with > 60% of total metabolites[187]. Zhao *et al*[136] reported that the LCMs are unconjugated in feces. Because of the lack of β-glucuronidase treatment, Jiang *et al*[187] could not distinguish between conjugated and non-conjugated metabolites in their fecal samples, which in turn could support the results from Zhao *et al*[136]. Wu and Croft[188] concluded that part of the total vitamin E undergoes enterohepatic circulation (estimated to be about 60% in rats) and that the remaining is likely lost *via* the fecal route[189]. However, the fecal portion of metabolite excretion was estimated to be about 80%[161]. As mentioned before, intestinal absorption was reported to be between 20%-80% for α-TOH, leaving a considerable portion that remains in the intestinal tract[188]. Zhao *et al*[136] tested whether intestinal flora is able to degrade

TOHs by incubating fecal extracts with TOHs, but this experiment failed to produce vitamin E metabolites. To summarize, SCMs are transported *via* blood and are excreted through urine, while TOH, T3 and all other metabolites circulate through the vascular system (shown for α-13'-COOH in humans by our group[142]) and are secreted into bile and eliminated *via* feces.

Thus far, no specific transport or binding proteins for CEHCs or CEHC conjugates have been reported. However, some phase Ⅲ transporters are involved in the elimination of vitamin E, or their expression is regulated by vitamin E[63]. Early findings by Bjørneboe *et al*[190] in rats injected with radioactively labeled α-TOH revealed that 14% of the radioactivity was recovered during 24 h of bile draining, thus indicating an involvement of biliary excretion pathways. Since then, the underlying molecular mechanisms have been of particular interest. Apob seems to be involved in the secretion of α-TOH from liver cells into blood, as in *Apob*-knockout mice the biliary secretion of α-TOH is significantly decreased after a single *i.v.* injection of 25 mg/kg α-TOH compared to the controls[191]. Mardones *et al*[82] provided evidence that SR-B1 encoded by the *Scarb1* gene contributes to biliary excretion of α-TOH, as the hepatic concentration of α-TOH is normal compared to the controls but biliary excreted α-TOH was 74%-81% lower in *Scarb1*-knockout mice. Expression of SR-B1 is also reported to be regulated by α-TOH. Mice kept vitamin E-deficient showed elevated hepatic SR-B1 protein levels, which were reversible by feeding them a vitamin E-enriched diet, whereas HepG2 cells cultured in the presence of vitamin E-loaded HDL showed decreased SR-B1 levels[192]. According to Takada and Suzuki[193], SR-B1 might be responsible for the import of vitamin E into hepatocytes when located at basolateral membranes and also for the export into bile when expressed at the canalicular site. Therefore, many studies have aimed to clarify the differing expression patterns of SR-B1 between sinusoidal and canalicular membranes[194]. Comparison of human and mouse liver tissues as well as HepG2 cells have revealed pronounced differences. While *ex vivo* SR-B1 was highly enriched in sinusoidal membranes and was also found in canalicular membranes, HepG2 cells clearly showed enrichment of SR-B1 in bile canalicular-like structures[194].

Multidrug resistance proteins (MDR) are also involved in the elimination of α-TOH, as inhibition or deletion of canalicular Mdr2 (also named Ppg or p-glycoprotein) leads to a decrease in the basal release of α-TOH into bile in rats and mice[189]. Results from *in vitro* studies on MDR1 did not reveal clear results. Primary human hepatocytes showed no response in the expression of MDR1 to T3 treatment, whereas intestinal LS180 cells reacted with a clear increase of MDR1 expression (depending on the type of T3)[180]. In line with the latter finding, rats daily injected subcutaneously with 10 mg/100 g body weight α-TOH had increased levels of hepatic Mdr1 protein beginning at day 9 and reaching a peak at day 15 of injections[167]. Further, mice fed with 1000 IU/d *all-rac*-α-TOH acetate for four months showed elevated levels of Mdr1a protein, the mouse orthologue of human MDR1, compared to the controls, while expressions of Mdr1b, Mdr2, Abcc2, Abcc6 and breast cancer resistance protein 1 (Bcrp1/Abcg2) remained unchanged[183]. When rats were daily injected subcutaneously with 100 mg/kg body weight α-TOH, the hepatic efflux transporters Abcb1b and Abcg2 were upregulated, while the organic anion transporting polypeptide 2 (Oatp), a liver influx transporter, was downregulated[195]. Traber *et al*[195] noted that Abcg2 transports sulfates and glucuronides and suggested that this protein is involved in the excretion of conjugated vitamin E metabolites, such as CEHCs, while Oatp might be involved in the uptake of CEHCs into liver cells. Summarizing the current knowledge on excretion of vitamin E metabolites, it has to be emphasized that many aspects regarding the involvement of transporters in vitamin E and vitamin E metabolites still lack clarity. It remains to be resolved whether and which specialized proteins for the regulated excretion of vitamin E metabolites exist.

Kinetics of vitamin E degradation: All vitamin E forms are in principle degraded *via* the same pathways independent from their substitution pattern with methyl groups at the chromanol ring system. However, the rate of degradation depends on the methylation pattern of the chromanol ring, the saturation of the side-chain, and on the source of vitamin E (*i.e.,* natural *vs* synthetic).

Natural forms of vitamin E, *i.e.,* enantiopure vitamin E forms with the *RRR* configuration, are partially protected from degradation compared to synthetic forms, *i.e.,* vitamin E forms with the *all-rac* configuration, as was shown by Traber *et al*[196], who supplemented humans with 150 mg d_3-*RRR*-α- or d_6-*all rac*-α-TOH acetates. While d_3-*RRR*-α-TOH accumulated in plasma, d_6-α-CEHC derived from d_6-*all rac*-α-TOH was found almost only in urine. Vitamin E forms with unsaturated side-chains are degraded faster than the saturated forms. T3 were reported to appear in human plasma with half-lives of 4.3, 4.4, and 2.3 h for α-, γ- and δ-T3, respectively[23], whereas for *RRR*-α-TOH a half-life of 45 h[110] to 60 h[197] was found. When humans were supplemented with a single dose of 125 mg or 500 mg γ-T3, urinary excretion levels of γ-CEHC rose about 4- to 6-fold with a maximum at 9 h after ingestion and a decline to baseline by the following day[198]. An increase of urinary α-CEHC was only observed after ingestion of a very high dose of α-tocotrienyl acetate (500 mg compared to 125 mg)[198]. This study found 1%-2% of α-tocotrienyl acetates and 4%-6% of γ-tocotrienyl acetates as urinary metabolites, suggesting alternative elimination routes for T3s[198].

As outlined above, the elimination of vitamin E forms depends on the methylation pattern of the chromanol ring system. Zhao *et al*[136] analyzed human serum after

one-time supplementation with a mixture of different vitamin E forms (2400 mg γ-TOH, 1596 mg α-TOH, 936 mg δ-TOH and 24 mg T3s) and found concentrations of α-, γ- and δ-TOHs of 21.1, 6.19, and 0.5 μmol/L, respectively, as well as ICM and SCM concentrations after enzymatic hydrolysis of 0.03 μmol/L (α-5′-COOH), 0.21 μmol/L (γ-5′-COOH), 0.08 μmol/L (δ-5′-COOH), 0.02 μmol/L (α-CEHC), 0.35 μmol/L (γ-CEHC), 0.09 μmol/L (δ-CEHC) 12 h post dose. In support of these findings the estimated half-life of γ-TOH is, at 12 ± 4 h[197], shorter than that of α-TOH at 45 h[110] to 60 h[197]. Leonard *et al*[197] administrated humans orally with about 50 mg of an equimolar mixture of d_6-α-TOH and d_2-γ-TOH acetates and found no increase of α-CEHC (detection limit in this study: 1 nmol/L), while γ-CEHC plasma concentrations doubled (129 ± 20 to 258 ± 40 nmol/L in women) after 12 h. Schuelke *et al*[116] concluded that α-CEHC excretion follows α-TOH plasma levels only when a threshold of 30-40 μmol/L α-TOH in plasma is exceeded. Rats treated orally with an oil containing 10 mg of γ-TOH or a combination of α- and γ-TOH (10 mg each) had urinary γ-CEHC levels which reached their highest levels 24 h to 30 h post application in both groups, but the concentration of γ-CEHC was 20%-50% higher in the α- and γ-TOH treated group compared to the group receiving only γ-TOH[149]. In addition, a shift from biliary to urinary excretion of γ-CEHC was observed, with γ-CEHC concentrations being higher for the combined treatment with α- and γ-TOH than for the γ-TOH-treated group[149]. Analyzing the absolute contents of γ-CEHC in bile and urine, they found 130 μg or 190 μg for γ-TOH or α- and γ-TOH in bile, respectively, and 250 μg γ-CEHC in the γ-TOH treated group and 280 μg in the α- and γ-TOH treated group. The authors therefore suggested that the major excretion pathway for γ-CEHC is *via* urine[149]. Mustacich *et al*[167] injected rats daily with 10 mg/100 g body weight α-TOH and analyzed the hepatic concentrations of LCM α-13′-OH and ICM α-5′-COOH. The study revealed an increase in both metabolites for the first measurement after three days post first application, with higher concentrations for α-13′-OH (up to 6.4 ± 0.7 nmol/g; 20-fold higher than prior to injection) than for α-5′-COOH (1.0 ± 0.3 nmol/g at day 3; undetectable prior to injection). None of the other known metabolites were found in the analyzed livers. The levels of α-13′-OH decreased to 1.2 ± 0.2 nmol/g at day 18 and levels of α-5′-COOH decreased to 0.4 ± 0.1 nmol/g at day 12; both values remained unaffected by the subsequent injections of α-TOH. Bardowell *et al*[157] speculated that mechanisms other than ω-hydroxylation might contribute to the elimination of non-α-TOH forms of vitamin E. They proposed formation and fecal excretion of ω-1 and ω-2 metabolites γ-TOH, namely γ-12′-OH and γ-11′-OH, as well as fecal elimination of non-metabolized TOH, as these metabolites and their precursor TOHs were found in human feces. Zhao *et al*[136] found several vitamin E metabolites in mouse feces (see Table 1) after supplementation with a diet enriched with 0.3% of a mixture of different vitamin E forms (20.2% α-T3, 4.0% β-T3, 16.1% γ-T3, 9.9% δ-T3, 14.8% α-TOH, and 3.1% γ-TOH) for four weeks and reported increases in the concentrations of almost all of these metabolites in human fecal samples over time after a single dose supplementation of 2400 mg γ-TOH, 1596 mg α-TOH, 936 mg δ-TOH und 24 mg T3s. To sum up, elimination of non-α-TOH forms is not only greater than of α-TOH, but α-TOH also increases elimination rates of non-α-TOH forms of vitamin E. This can be explained by the physiological action of α-TTP (see the corresponding section on α-TTP in chapter "Intracellular binding proteins") and vitamin E ω-hydroxylase CYP4F2 (see the section on "Enzymatic degradation of vitamin E").

Parker *et al*[171] published a hypothesis to explain the underlying physiological importance of the different rates of metabolism and elimination of the different forms of vitamin E. When murine macrophages (RAW264.7 cells) were incubated with α-TOH, γ-TOH, δ-TOH or δ-T3, different cell viabilities were found: Cell viability for α-TOH was not impaired but was intermediately reduced for γ-TOH and cell viabilities for δ-TOH or δ-T3 were substantially lower. The catabolism rates of these vitamin E forms in HepG2 cells, however, were inverse, as almost no metabolites for α-TOH but increasing amounts of metabolites for the other vitamin E forms investigated were found, with highest amounts for δ-T3. Therefore, the authors suggested an inverse correlation between the different cytotoxicities of the different vitamin E forms and their elimination rates.

Gender-specific bioavailability of vitamin E: Several years ago, differences in the bioavailability of vitamin E between genders were described. Feingold *et al*[199] observed in rats that α-TOH levels were higher in adrenal glands and livers as well as in all subcellular fractions derived from female rats compared to male rats. However, no sex differences in the subcellular distribution of α-TOH were observed. Notable differences in the kinetics of vitamin E metabolism were observed also in humans orally administered with a single dose of 50 mg deuterium-labeled d_6-α-TOH acetate and 50 mg d_2-γ-TOH acetate. Women showed higher maximum plasma concentrations of d_2-γ-CEHC and excreted four times more d_2-γ-CEHC in urine as men[197]. However, in both sexes urinary d_2-γ-CEHCs concentrations were decreased within 24 h after consumption of sesame oil, containing sesamine, a known inhibitor of vitamin E metabolism[200]. In line with this, urinary excretion of non-deuterated γ-CEHC was also higher in women compared to men. But, in contrast to d_6-α-TOH, urinary excretion of non-deuterated α-CEHC was also higher in women than in men. This suggests that women have faster γ-TOH disappearance rates in plasma than men[200], possibly because of their higher HDL TOH levels[197]. In contrast to γ-TOH, no differences in plasma disappearance rate have been found for α-TOH. But,

serum concentrations of α-TOH are higher in females compared to men[33,39].

As postulated by Miwa *et al*[39], the observed gender-specific differences in α-TOH/lipids ratio in serum may be due to effects of the female hormone estrogen. Unfortunately, studies measuring serum vitamin E concentrations in humans have revealed contradictory results. In the SU.VI.MAX cohort of middle-aged French participants, lower serum vitamin E concentrations in women than in men were found[27]. But, when serum vitamin E levels were compared to energy intake or serum cholesterol concentrations, which are both lower in women than in men, vitamin E density per kcal intake was higher in women[27]. Reasons for the gender-specific differences in vitamin E serum levels in humans are most likely: (1) the hormonal differences; and (2) gender-dependent differences in the activation of the CYP enzymes involved in vitamin E metabolism[201]. In many species, expression of liver-expressed genes depends on hormones and growth factor profiles, which show sexual dimorphism[202-206]. For example, the transcription factor Stat5b is a key factor in maintaining the sexual dimorphic response of CYP gene expression[202,207]. As a consequence, protein levels of CYP enzymes, such as Cyp4f1, Cyp4f4, Cyp4f5 and Cyp4f6, are usually higher in liver, kidneys, lungs and brain of female rats compared to male rats[208]. In the case of the CYP4F family, Kalsotra *et al*[208] suggested the importance of estrogen in regulating the sex-specific expressions of Cyp4f1, Cyp4f4 and Cyp4f6 in the kidneys and liver of rats. It is questionable whether the sex-specific differences in hepatic CYP expression profiles can be directly transferred to the case of humans. Whereas sex differences in drug metabolism have been shown in humans, too, larger interindividual differences in CYP activity have been also discussed[201,202]. Indeed, in general studies have shown that CYP3A activity is higher in women than in men[201]. In line with the observation that estradiol and testosterone regulate the expression of CYP3A isoforms in the liver[209], Parkinson *et al*[210] and Wang *et al*[204] demonstrated that women metabolize some CYP3A4 and CYP3A9 substrates faster than men do.

It is known that vitamin E levels change under metabolic challenges, such as age-associated diseases or oxidative stress (see the section "Life-style and age influence the bioavailability of vitamin E" in the chapter on the bioavailability of vitamin E). Furthermore, sex-related differences occur under these conditions, as observed by Aryamanesh *et al*[211]. This group described increased serum levels of α-TOH in women compared to men under ischemic preconditioning. Cavalca and colleges[212] found interactions between coronary artery disease (CAD) status, gender and α-TOH concentrations; in this study α-TOH concentrations were lower in female CAD patients than in male patients. Further analysis revealed that women with CAD, but not men, had lower serum levels of α-TOH compared to women with no CAD. Interestingly, no gender-specific differences were observed for γ-TOH[212].

Extrahepatic vitamin E metabolism: In addition to the established importance of hepatic metabolism of vitamin E, there is evidence for extrahepatic, namely intestinal vitamin E catabolism. First hints were obtained from studies by Abe *et al*[164], in which ketoconazole decreased γ-CEHC concentrations in jejunum after oral gavage of γ-TOH. A preferential degradation of γ-TOH and γ-T3 over α-TOH was also found. Further evidence was provided by Bardowell *et al*[157], as mice lacking hepatic microsomal CYP enzyme activity (including Cyp4f14) had only 70% reduced vitamin E metabolism after supplementation with γ- and δ-TOH instead of the expected total absence of metabolites. Supporting this observation, Cyp4f14 is expressed in the small intestine[213] and ω-hydroxylase activity for several forms of vitamin E was found by Bardowell *et al*[157]; this activity comprised about 10% of the ω-hydroxylase activity found in the liver. Mice deficient for Cyp4f14 had no ω-hydroxylase activity, leading to the conclusion that the small intestine, in contrast to the liver, may have only one enzyme capable of hydroxylating vitamin E[157].

GENETIC POLYMORPHISMS

Interindividual differences in vitamin E metabolism are known and have been reported for, for example, γ-CEHC excretion after γ-TOH supplementation[198], as well as for α-TOH metabolism[176]. Both studies suggest that differences in the absorption and metabolism of vitamin E are responsible for these effects. In agreement with this suggestion, genetic polymorphisms are known for genes involved in vitamin E homeostasis, which could cause such interindividual differences. An overview on known polymorphisms in genes encoding for proteins involved in vitamin E homeostasis is provided in Table 3.

Döring *et al*[214] were the first to systematically evaluate single nucleotide polymorphisms (SNPs) in genes encoding for proteins involved in vitamin E homeostasis. They analyzed *TTPA, TAP, LPL, ABCC3/MRP2, PXR* and *CYP* genes such as *CYP3A4, CYP3A5* and *CYP4F2* for the frequency of SNPs in coding regions and found *LPL*, *ABCC3/MRP2, PXR, CYP3A4* and *CYP4F2* to be highly polymorphic in their coding region. Recently, a new screening for candidates was published by Borel *et al*[215]. The postprandial chylomicron α-TOH response was positively correlated to fasting plasma α-TOH levels and 28 SNPs in eleven genes were identified that may contribute to 82% of the postprandial chylomicron α-TOH response, namely *ABCA1, ABCG1, APOB, BET1, IRS1, LIPC, NAT2, PNLIP, SLC10A2, SREBF2* and *ZNF664*. Among these genes are seven which are involved in chylomicron metabolism and are therefore indirectly responsible for vitamin E transport in serum. Borel *et al*[215] noted that *TTPA, CD36, SCARB1* and *SEC14L* were not found to be associated with the response of serum α-TOH levels in this analysis.

Table 3 Genetic polymorphisms of genes encoding for proteins involved in vitamin E homeostasis

Gene	Polymorphism	RefSNP ID	Functional consequence	Ref.
ABCA1	NM_005502.3:c.2828+426A > G	rs4149314	Intron variant	[215]
	NM_005502.3:c.814-1304T > C	rs4149297	Intron variant	[215]
	NM_005502.3:c.161-170G > A	rs11789603	Intron variant	[215]
	NM_005502.3:c.936C > A	rs2274873	Synonymous codon	[215]
	NM_005502.3:c.936C > T			
ABCG1	NC_000021.9:g.42167452C > T	rs468320		[215]
APOA5	NM_052968.4:c.-644C > T	rs662799	Upstream variant 2KB	[219]
APOB	NC_000002.12:g.20937665C > T	rs4643493		[215]
	NM_000384.2:c.12541G > A	rs1042031	Intron variant Missense: E4181K	[215]
	NC_000002.12:g.21048451A > G	rs1713222		[215]
BET1	NC_000007.14:g.94352727G > A	rs10464587	Intron variant	[215]
CD36	NM_001289911.1:c.-108-13288T > C	rs1527479	Intron variant	[220]
CYP4F2	NM_001082.4:c.1297G > A	rs2108622	Missense: V433M	[217,225]
	NM_001082.4:c.34T > G	rs3093105	Missense: W12G	[225]
IRS1	NC_000002.12:g.226683594A > G	rs1316328		[215]
LIPC	NC_000015.9:g.58688186A > C	rs4238329		[215]
	NM_000236.2:c.89-47937A > G	rs8041525	Intron variant	[215]
	NC_000015.10:g.58394555C > T	rs7164909	Intron variant	[215]
	NC_000015.10:g.58353522T > C	rs8035357	Intron variant	[215]
	NM_000236.2:c.88+49790T > C	rs12591216	Intron variant	[215]
	NM_000236.2:c.89-50891C > A	rs12593880	Intron variant	[215]
NAT2	NC_000008.11:g.18439195T > C	rs4921920		[215]
NPC1L1	NM_013389.2:c.1184C > T	rs62001882	UTR variant 5 prime Missense: A395V	[216]
	NM_013389.2:c.1204G > A	rs141973731	UTR variant 5 prime Missense: G402S	[216]
	NM_001300967.1:c.1249C > T	rs139659653	UTR variant 5 prime Missense: R417W	[216]
	NM_013389.2:c.1300G > A	rs114375162	UTR variant 5 prime Missense: G434R	[216]
PNLIP	NM_000936.2:c.930+205T > C	rs2915775	Intron variant	[215]
	NC_000010.11:g.116569152G > T	rs3010494		[215]
SCARB1	NM_001082959.1:c.127-4800C > T	rs11057830	Intron variant	[217]
SEC14L2	NM_033382.2:c.32G > A	rs757660	UTR variant 5 prime Missense: R11K	[224]
	NM_016498.4:c.-2240T > C	rs1061660	Upstream variant 2KB, UTR variant 3 prime	[224]
	NM_016498.4:c.-2110G > A	rs1061664	Upstream variant 2KB, UTR variant 3 prime	[224]
SLC10A2	NC_000013.11:g.103037119C > T	rs1571513		[215]
	NC_000013.11:g.103898490C > T	rs9558203		[215]
	NC_000013.11:g.103040492G > A	rs16961116		[215]
	NC_000013.11:g.103444248G > A	rs12874168		[215]
	NC_000013.11:g.103452026G > A	rs2065550		[215]
SREBF2	NM_004599.3:c.2208+3245C > T	rs2839715	Intron variant	[215]
	NM_004599.3:c.1761+160G > A	rs4822062	Intron variant	[215]
TTPA	NM_000370.3:c.421G > A	rs397515524	Intron variant Missense: E141K	[222]
	NM_000370.3:c.175C > T	rs397515522	Missense: R59W	[222]
	NM_000370.3:c.-1753G > A	rs12056582	Upstream variant 2KB	[223]
	NM_000370.3:c.-1410A > T	rs6472071	Upstream variant 2KB	[223]
	NM_000370.3:c.-1410A > C -345C/T[1]	rs75371508		[223]
	NM_000370.3:c.-981T > A	rs6994076	Upstream variant 2KB	[223,224]
	NM_000370.3:c.-945G > A	rs34358293	Upstream variant 2KB	[223]
	NM_000370.3:c.-675G > A	rs80169698	Upstream variant 2KB	[223]
	NM_000370.3:c.-440C > T -344C/T[1]	rs73684515 rs74684018	Upstream variant 2KB	[223] [223]
ZNF664	NM_001204299.1:c.-234+53341T > C	rs7296124	Intron variant	[215]
	NM_152437.2:c.*2065G > A	rs1048497	Intron variant, UTR variant 3 prime	[215]
ZPR1	NM_003904.3:c.*724C > G	rs964184	Downstream variant 500B	[217]

[1]This variant has been described by Ulatowski *et al*[223] but was not found in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

The importance of variants of a transporter involved in the intestinal uptake of lipids and lipophilic nutrients, namely NPC1L1, was analyzed *in vitro* and four variants, located on the predicted extracellular loop of NPC1L1

protein (*i.e.*, A395V, G402S, R417W, and G434R) showed lower transport efficiencies for both cholesterol and α-TOH[216]. Borel *et al*[217] analyzed polymorphisms in genes involved in lipid metabolism, namely *APOA4, APOB, APOE, LPL* and *SRBI*, and found α- or γ-TOH levels to be different in subjects with SNPs in *APO4, APOE* and *SRBI* or *APO4* and *SRBI*, respectively. Major *et al*[218] found three SNPs to be associated with circulating α-TOH serum levels, rs964184 near *APOA1*, *APOA4*, *APOA5* and *APOC3*, rs2108622 in *CYP4F2* as well as rs11057830 in *SCARB1*. As the importance of proteins involved in lipoprotein metabolism for vitamin E transport and serum levels has been highlighted by these findings, Sundl *et al*[219] emphasized that vitamin E serum levels should be adjusted for plasma or serum lipids. The authors reported that the association of the APOA5 -1131T>C variant (rs662799) with vitamin E serum concentrations was no longer statistically significant, when the vitamin E values were adjusted for serum triglyceride or total serum cholesterol concentrations. The scavenger receptor cluster of differentiation 36 (CD36) might also be involved in cellular vitamin E uptake[220], and indeed a SNP (rs1527479) was identified to be associated with lower α-TOH serum concentrations[221].

As already mentioned, hepatic α-TTP plays a central role in vitamin E homoestasis. As some genetic variants in the *TTPA* gene encoding α-TTP cause reduced circulating α-TOH levels in serum, Bromley *et al*[222] analyzed two SNPs in the *TTPA* gene, uncovering the variants E141K and R59W, which are known to be associated with AVED. These variants are located in or close to the proposed ligand-binding domain of α-TTP, and the authors showed that the variants are responsible for reduced binding of α-TOH to α-TTP *in vitro*. Ulatowski *et al*[223] analyzed SNPs in the promotor region of the *TTPA* gene and found that the substitutions -1752C/T (rs12056582), -1408G/T (rs6472071), and -345C/T (rs75371508) increased promotor activity about 3-fold, whereas the SNPs -1408A/T (rs6472071), -980A/T (rs6994076), -943A/G (rs34358293), -674C/T (rs80169698), -439A/G (rs73684515), and -344C/T (rs74684018) repressed promoter activity. To support the physiological relevance of their findings the authors noted that Wright *et al*[224] found reduced vitamin E plasma levels to be associated with the SNP -980A/T in the promotor region of the *TTPA* gene. In addition, three SNPs in the coding region of the *SEC14L2* gene encoding TAP1 are associated with increased serum vitamin E concentrations[224]. Major *et al*[218] identified a new SNP in the *CYP4F2* gene (rs2108622) which encodes the vitamin E ω-hydroxylase. The physiological relevance of this SNP and the SNP rs3093105 has been characterized by Bardowell *et al*[225]. The CYP4F2 W12G variant leads to a 2.3 to 2.8-fold increased specific enzyme activity for both TOHs and T3s, whereas the CYP4F2 V433M (rs2108622) variant causes reduced enzyme activity for TOHs, without affecting the activity for T3s. Athinarayanan *et al*[226] analyzed the impact of these two genetic variants on vitamin E plasma levels in children and adults with non-alcoholic fatty liver disease. The study revealed moderate effects on plasma vitamin E levels during vitamin E treatment only for the V433M variant but showed no effects on histological parameters, such as fibrosis, ballooning or steatosis.

In summary, genes involved in vitamin E homeostasis are polymorphic and some genetic variants, in particular in coding and promotor regions, are associated with vitamin E plasma/serum levels.

METABOLIC INTERACTIONS WITH VITAMIN E CATABOLIC PATHWAY

As the pathways responsible for vitamin E metabolism are not restricted to vitamin E, interactions with other metabolic pathways can occur that make use of: (1) the same enzymes; (2) the vitamin E-dependent regulation of gene expression; or (3) the enzyme activity of xenobiotic pathways. Two possible categories of interactions are discussed here; firstly, interaction with vitamin K metabolism and secondly, with xenobiotics and drugs.

Interaction with vitamin K metabolism

The interference of vitamin E with vitamin K metabolism and, as a result, with blood coagulation has been known for decades[227]. As shown by several supplementation studies with vitamin E in rats[228] or humans[229] an increased risk of bleeding was noted. The underlying molecular mechanisms are still unclear, but recent research has shed new light on this aspect of vitamin E metabolism.

Vitamin E and vitamin K share the same metabolic pathways, as the degradation of both vitamins is initiated with an ω-hydroxylation followed by subsequent β-oxidation of the aliphatic side-chain, thus resulting in urinary and biliary excretion of the respective carboxylic acids or conjugates with shortened side-chains[230]. When vitamin E increases the expression or activity of enzymes involved in its own degradation, it is possible that vitamin K metabolism is also enhanced under elevated vitamin E status; this may lead to higher rates of vitamin K excretion and in turn to vitamin K deficiency with enhanced bleeding risk[160]. Evidence for this hypothesis was provided by Hanzawa *et al*[231], as rats treated with a diet consisting of 20% sesame seeds, containing sesamin as an inhibitor of vitamin E metabolism, for 40 d had increased vitamin K tissue concentrations. It should be noted that the vitamin E form used in this diet was solely γ-TOH. Farley *et al*[232] investigated the influence of α-TOH on phylloquinone or menadione metabolism and excretion. They fed rats with either a phylloquinone or a menadione containing diet (2 μmol/kg body weight) for 2.5 wk and injected after ten days a daily dose of 100 mg/kg body weight α-TOH for a further seven days. Tissue levels of menaquinone-4, a tissue-specific metabolite of phylloquinone and menadione, were decreased in brain, lung, kidney and heart and levels of phylloquinone were

decreased in lung independent of the type of diet. The authors observed a downregulation of the expression of CYP enzymes CYP3A, CYP4F4 and CYP4F1, which was explained by an alternative mode of interference of vitamin E with vitamin K metabolism apart from the induced degradation of vitamin K. The induction of the xenobiotic exporters ABCB1/MDR1 and ABCG2/BCRP1 provided the first hints for an increased excretion of vitamin K metabolites into bile. This concept is supported by the 100-fold increased urinary excretion of α-CEHC in response to the application of α-TOH, whereas urinary excretion of vitamin K metabolites remained unchanged. In another study, Farley *et al*[233] focused on CYP4F2, as this protein is involved in the degradation of both vitamin E[140] and vitamin K[234]. The catalytic efficiency of CYP4F2 for the hydroxylation of phylloquinone is higher than for α-TOH, while the co-incubation of phylloquinone and α-TOH had no influence on the metabolism of phylloquinone. Thus, this study indicates that the activity of CYP4F2 is not enhanced by high concentrations of α-TOH. In their most recent study, Farley *et al*[235] concluded that α-TOH, or even α-CEHC, interferes with an intermediate step in the formation of tissue-specific menaquinone-4 from phylloquinone, such as alterations in the transport *via* chylomicrons or other lipoproteins or reductions of the cellular uptake of phylloquinone or menadione, an intermediate product of phylloquinone in the menaquinone formation pathway. This hypothesis is supported by findings of Hanzawa *et al*[236] who found that rats fed a diet with 0.75 mg/kg phylloquinone for six weeks have decreased phylloquinone tissue concentrations with supplementation of 100 mg α-TOH while in rats fed with 0.75 mg/kg menaquinone diet the tissue concentrations of menaquinone remained unchanged with an increased application of α-TOH.

Finally, the form of vitamin E also contributes to the interference with vitamin K metabolism, as γ-TOH is not as potent as α-TOH in decreasing extrahepatic phylloquinone concentrations[236]. Taken together, vitamin K metabolism is disturbed by vitamin E at the level of extrahepatic synthesis of menaquinone from phylloquinone or menadione and the different vitamin E forms also lead to differing effects on the metabolic interactions.

Interactions with pharmaceutics

As CYP3A4 is responsible for the metabolism of more than 50% of xenobiotics[237], the upregulation of CYP3A4 expression by α-TOH may be of concern regarding the interactions of α-TOH with drug metabolism. Clarke *et al*[238] investigated the effect of α-TOH on midazolam plasma concentrations and found no influence. When the interaction with cyclosporine A was analyzed, Bárány *et al*[239] found reduced plasma levels after six weeks of supplementation with 800 IU/d α-TOH. These results indicate possible interactions of α-TOH with drug metabolism that need further investigation.

Vitamin E was a promising candidate as a supplement to prevent atherosclerosis and its complications. Data obtained from *in vitro* studies have been very promising, yet large intervention studies have revealed inconsistent results (for an overview, see Wallert *et al*[240]). There may be many reasons to explain this finding, but the details still need to be unraveled. With respect to patients who take medications, the situation may be even more complicated. For example, in patients treated with statins to reduce LDL cholesterol levels, interactions with simultaneously ingested vitamin E supplements may occur. A combined treatment with atorvastatin and α-TOH in stable, non-diabetic dialysis patients revealed a reduced *in vitro* oxidizability of LDL[241]. Podszun *et al*[242] found no interactions in guinea pigs fed with high-dose α-TOH (250 mg/kg diet) for six weeks when atorvastatin (300 mg/kg diet) was simultaneously applied. In 2001, however, two studies were published which questioned the benefit of the combined therapies. Patients treated with simvastatin, niacin and a combination of different antioxidants (400 IU *bis in die* (BID; twice a day) vitamin E, 500 mg BID vitamin C, 12.5 mg BID β-carotene and 50 mg BID selenium) profited less than the simvastatin and niacin groups as the protective increase in HDL_2 with simvastatin plus niacin was attenuated by concurrent therapy with antioxidants[243,244]. Tousoulis *et al*[245] treated patients suffering from ischemic heart failure with low dose atorvastatin (10 mg/d) alone or in combination with 400 IU/d vitamin E and found that vitamin E impairs the positive effects of atorvastatin, on, for example, endothelial function or inflammatory response.

Many studies focusing on the effects of statins on human lipid levels also analyzed other plasma parameters, such as vitamin E status. The results depend, among others, on the evaluation of the data. When vitamin E serum levels were measured without any corrections most researchers found a decrease[241,246-250]; however, when vitamin E serum levels were normalized to plasma lipids, such as LDL cholesterol, total serum cholesterol or serum triglycerides, increased or unchanged ratios of vitamin E and serum lipids were observed[247,249-252]. The underlying processes are still unclear but on the one hand, the decrease of circulating lipoproteins could directly cause reduced levels of vitamin E (in terms of absolute concentrations), whereas the ratio of vitamin E to serum lipids could at the same time be increased, as statins mostly decrease specific lipid fractions, namely LDL cholesterol and total cholesterol. On the other hand, Werba *et al*[253] suggested an inhibitory effect of some statins, such as simvastatin, on CYP3A4.

VITAMIN E STATUS

When assessing vitamin E status, analytical parameters which have proven the most reliable must be primarily considered. As noted by Traber[18] in 2014, circulating α-TOH concentrations are not necessarily a reliable marker for an adequate vitamin E status in humans.

Supplementation of α-TOH, for example, increases serum levels but leads simultaneously to increased amounts of α-CEHC in urine. Hence, α-CEHC in urine can be used as a marker for α-TOH status in healthy humans[132] or at a minimum as a marker for an adequate level of α-TOH[116]. In addition to serum α-TOH levels and urinary α-CEHC, the quest for another more reliable marker for adequate supply of vitamin E would be helpful for determining vitamin E status. Our group has been the first to reveal preliminary evidence for the systemic bioavailability of the first hepatic metabolites, α-13'-OH and α-13'-COOH, and their extrahepatic presence[142]. We suggest that the serum concentrations of these α-LCMs may be promising candidates in the search for new reliable markers of α-TOH levels. Both LCMs increase after supplementation with 1000 IU *RRR*-α-TOH for a few days. It should be also noted that the assessment of absolute food and energy intake is needed when vitamin E concentrations are measured and correlated to gender, age and life-style[118]. Determining the right analytical parameters for evaluating vitamin E status is critically important; however it is also crucial that new analytical parameters and procedures be validated, optimized and standardized for ensuring optimal diagnosis and comparability.

Analytics

The analytics here involve numerous elements which should be considered before beginning measurements. Yu *et al*[254] demonstrated nicely that the reproducibility of the measurement of vitamin E metabolites is better in plasma than in serum samples, while the concentrations measured in serum are higher. The sample matrix also matters in the determination of conjugated metabolites. Freiser and Jiang[141] showed that conjugated metabolites are more difficult to hydrolyze in plasma compared to other biological fluids, such as urine. In principle, there are two methods for analyzing conjugated metabolites, the simultaneous analysis of conjugated and non-conjugated metabolites or the pre-treatment of samples with the respective enzymes glucuronidase and sulfatase[139]. With respect to the simultaneous analysis, Jiang *et al*[139] stated that "neither electrochemical detection nor GC-MS can be used to detect sulfated carboxychromanols because of their lack of redox activity or the inertness of the conjugated phenolic group to chemical derivatization, respectively". Pope *et al*[177] developed the first tandem MS-MS approach for the reliable and simultaneous detection of conjugated and non-conjugated metabolites. Since then, the analytical procedures for measuring the metabolites in both conditions were adopted for LC-MS[135] and UPLC-TOF-MS[176].

Treatment of samples with deconjugation enzymes must be performed carefully. Only a cleanup-procedure with methanol/hexane extraction for the removal of proteins and lipids in the sample and an enzymatic hydrolysis overnight ensures complete deconjugation; otherwise the conjugated CEHCs are significantly underestimated[141]. It must be considered that the length of the side-chain may also influence the efficiency of deconjugation, because sulfatase treatment of relatively hydrophilic compounds (*e.g.*, sulfated 9'-COOH) is more efficient than the treatment of more hydrophobic compounds (*e.g.*, sulfated 13'-COOH)[141]. Freiser *et al*[141] optimized deconjugation of vitamin E metabolites and found that only an overnight treatment with β-glucosidase/sulfates ensures optimal recovery. Although enzymatic hydrolysis is a standard procedure, the type of enzymes used differs[255]. In some studies recombinant enzymes with distinct activities are used, such as β-glucuronidase from *E. coli*[144,147,157], whereas others[149,151,154-156,177] used enzymes with the combined activity of β-glucosidase and sulfatase. In these cases, glucuronidase activity is usually higher than sulfatase activity. Here it is important to consider the study by Freiser *et al*[141] who reported that type B-1 β-glucuronidase from bovine liver has the lowest sulfatase activity of all enzymes tested, which is consistent with the common use of type H-1 enzymes from *H. pomatia*. Pope *et al*[177] noted that deconjugation of CEHCs may produce artefacts, such as a conversion of CEHC to tocopheronolactone. In addition, extraction conditions are important in terms of yield and recovery. According to Lodge *et al*[151], acidification and ether extraction ensure the highest recovery rates for α- or γ-CEHC from urine. To sum up, no standardized analytical method is available today which is suitable for use on all metabolites and conjugates. However, the establishment and careful evaluation of standardized analytics enables the reliable validation of biomarkers. This in turn offers the opportunity to obtain an accurate impression about the distribution and importance of vitamin E metabolites.

PERSPECTIVE

The coordinated mechanisms involved in the metabolism of vitamin E provide an effective but complex physiological pathway to protect the body against critical imbalances, *i.e.,* an excessive accumulation of non-α-TOH forms to unphysiological levels. However, vitamin E metabolism is complex and large gaps remain in the understanding of crucial processes which are involved. Thus far, the contribution of enzymes other than CYP enzymes and the mechanisms regulating the intracellular and interorgan trafficking and metabolism are still not understood in sufficient detail.

In addition to mechanistic insights into vitamin E metabolism, the physiological functions of the different vitamin E metabolites remains incompletely understood. While many researchers have focused in the past on the SCMs, there is a significant lack of knowledge on the physiological importance of the ICMs and, in particular, the LCMs. As of yet only a few studies have investigated the principle modes of action of the LCMs *in vitro*, while studies *in vivo* have not yet been performed as LCMs have not been available as pure compounds until

recently. As absolute concentrations of LCMs in the blood circulation of mammals are far too low for isolation, a natural compound, called garcinoic acid, isolated from the African bitternut *Garcinia kola* has proven to be a usefull resource to synthesize the α- and δ-LCMs of α- and δ-TOH[143]. Recent studies on α- and δ-LCMs have provided first preliminary insights[142,143,256-258] into the molecular modes of action of the LCMs, which seem to be specific and distinct from that of their metabolic precursors. We therefore hypothesize that at least some of the discrepancies in the results obtained from *in vitro* and *in vivo* studies on vitamin E might be explained by individual differences in the physiological metabolism of vitamin E, and in particular under pathological conditions. We suggest that the LCMs comprise a new class of regulatory metabolites, which must be investigated in more detail to unravel their proposed physiological relevance. In the meantime, a promising new approach may be to analyze serum concentrations of the LCMs in human intervention studies on vitamin E supplementation, to determine whether correlations exist to vitamin E intake and to the incidence of defined diseases.

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P- Reviewer: Cairo G
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6.7 Manuskript 7

Chapter 9

Garcinoic Acid: A Promising Bioactive Natural Product for Better Understanding the Physiological Functions of Tocopherol Metabolites

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Chapter Outline

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Studies in Natural Products Chemistry, Vol. 51. http://dx.doi.org/10.1016/B978-0-444-63932-5.00009-7

INTRODUCTION

Organisms produce bioactive natural products (secondary metabolites) as an adaption to their environment or as defense mediators. In contrast to primary metabolites such as protein, fat, and carbohydrates, they are not essential for growth, development, or reproduction [1,2]. Nevertheless, secondary metabolites are, like no other compounds, representatives for medical progress and have enormous importance for human health care. The use of natural products as medicines developed over generations and has been described throughout history in the form of folk medicine. The traditional African, Korean, Chinese, Islamic, and herbal medicines are the most important forms of historical folk medicine. Especially in Africa and Asia, 80% of the population still relies on traditional medicine for their primary health needs [3]. In these regions, fungi, plants, marine algae, or marine sponges are the most popular sources for bioactive natural products, but many of these compounds remain unexplored [2]. Nevertheless, plants are the dominant source of natural products in folk medicine. Plants have been well documented for their medicinal use for several thousands of years [4]. A well-known example is the plant *Alhagi maurorum*, which was used by the Romans for treating nasal polyps [5]. Plant-based traditional medicine was very important for primary health care over hundreds of years, but during the 18th century, the understanding of medicine changed. After Leeuwenhoek identified the first microorganism, enormous progress in the prevention of diseases was made. The knowledge associated with traditional medicine has promoted further investigations of compounds and extracts obtained from medicinal plants as potential medicines. This led to the isolation of many natural products from different sources.

One of the most famous examples is the antiinflammatory agent acetylsalicylic acid (aspirin) derived from the natural product salicin, which was isolated from the bark of the willow tree *Salix alba* [6,7]. During this

period, ethnopharmacological knowledge has been used for early drug discovery. Today, advances in analytical technologies improve the discovery of new bioactive natural products. These compounds have unique structural properties in comparison to products from standard combinatorial chemistry, making them the most promising source of lead structures for drug development [8,9].

A good example for the development from a medical plant used in traditional African medicine to a source of bioactive products for putative drugs is the African plant *Garcinia kola*. The parts of this plant contain many bioactive compounds, including the δ-tocotrienol (δ-T3) derivate garcinoic acid, which comprises an interesting molecule for functional studies. The aim of this review is to summarize the knowledge on this promising molecule and its use in research on vitamin E and its metabolites.

GARCINIA KOLA

G. kola or bitter kola is a dicotyledonous plant of the family *Clusiacea* (Fig. 9.1). It can be found in the rain forests of west and central Africa where it grows as a medium-sized tree with a height up to 12 m [10,11], but *G. kola* is also used for commercial farming, especially in Nigeria. The plant has reddish fruits containing two to four seeds. Both fruit components can be eaten [12]. *G. kola* plants bloom between December and January and their fruits mature from June to August [13]. From the botanical point of view, the fruits belong to the class of berries, but the seeds are often called *G. kola* nuts [14]. The nuts are dried and afterward available over the whole year, which gives them a small economical relevance [12]. Because of the bitter flavor of its seeds, the plant is colloquially called "bitter kola" or "bitter nut." The locals also name it "Orogbo" (Yoruba), "Aku ilu" (Igbo), and "Namijin goro" (Hausa) [3].

Apart from its small economical relevance, *G. kola* is very important for African ethnomedicine. Approximately 60–80% of the African population depend on herbal cures for their primary health care [3]. In the traditional African medicine, each part of the *G. kola* plant is used for different medical applications. For example, the root is used for oral hygiene and the tree bark as an abstergent agent. The latex of the tree is put on fresh wounds to prevent septic inflammation and to support healing [15]. The nuts are used for treating bronchitis and infections of the pharynx and colic [10]. Furthermore, the nuts are also used as antivenom for people with suspected intoxication [16]. It is also speculated that *G. kola* nuts protect against the toxic effects of alcohol [17]. Because of their bitter flavor, the bitter nuts are also used as stimulants for inducing anorexia [15]. Furthermore, antimicrobial effects [18–20], antiviral effects [10], antiparasite effects [21], antidiabetic effects [22], and hepatoprotective effects [23] have been described.

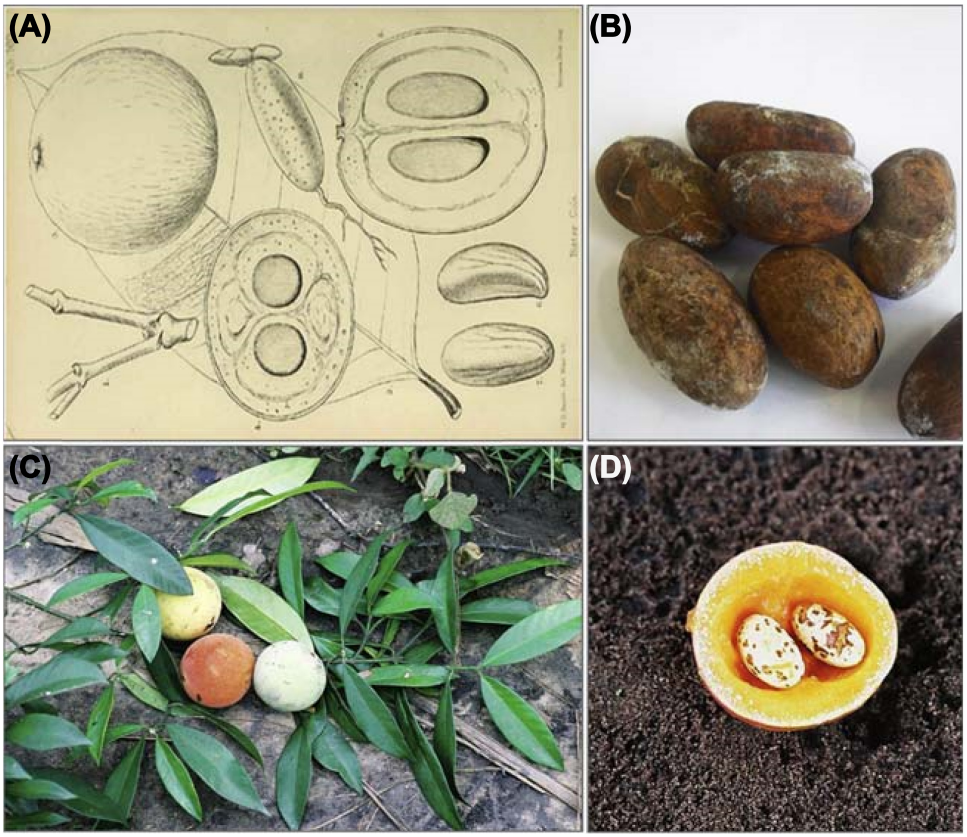

FIGURE 9.1 (A) Botanical illustration of the fruits of *Garcinia kola* E. Heckel *(Drawing by W.G. Smith published in 1875.)*. The fruits are shown completely and in cross section. Further, the seeds, colloquially called "bitter nut," can be seen. The illustration is entitled "bitter nut." (B) Photography of *G. kola* seeds. (C) Photograph of *G. kola* plant with fruits *(By courtesy of Paul Latham.)*. (D) Cross section of *G. kola* fruit, showing seeds *(By courtesy of Paul Latham.)*.

Bioactive Ingredients of the *Garcinia kola* Nut

The main components of *G. kola* nuts are carbohydrates, protein, fiber, fat, and water [3,12]. In contrast to the real kola nuts (*Cola nitida*), the bitter nuts do not contain caffeine [17], but they are a good source for calcium, potassium, sodium, and magnesium [3,24]. Furthermore, many other bioactive compounds, including tannins, saponins, alkaloids, and glycosides, have been isolated from *G. kola* nuts [3,13]. The nut also contains flavonoids and benzophenone derivatives such as kolaflavones and *Garcinia*-biflavones 1 (3,4,4,5,5,7,7-heptahydroxy-3,8-biflavanone) and 2 (3,4,4,5,5,5,7,7-hexahydroxy-3,8-biflavanone), which might be responsible for the observed antimicrobial effects of *G. kola* nuts [3]. Furthermore, two chromanols, garcinal and garcinoic acid, which have been described as strong antioxidants, have been isolated from *G. kola* seeds [25].

Biflavones and Benzophenone Derivatives

Most of the biochemical and physiological effects of the *G. kola* nut are attributed to its content of biflavones and benzophenone derivatives. One of the most investigated nut biflavones is kolaviron, a dimeric flavonoid (Fig. 9.2). In addition to its hepatoprotective effects [22] and its ability to lower blood cholesterol [26], antiinflammatory capacity has been shown for this compound in different animal models. For example, diabetic rats were supplemented with 100 mg/kg kolaviron for 6 weeks. The treatment with kolaviron resulted in a reduction of inflammatory processes, indicated by reduced serum concentrations of interleukin (IL)-1β and monocyte chemotactic protein 1 (MCP1) [27]. Similar results have been found in hepatic tissues of diabetic rats, where treatment with kolaviron reduced the amount of proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor α (TNFα) [28]. Further studies investigated the effects of kolaviron on inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) 2 expression in hepatic tissues of dimethylnitrosamine-treated rats. Dimethylnitrosamine is known as a hepatotoxin that enhances expression of iNOS and COX2 proteins as part of the proinflammatory response. After treatment with kolaviron, a significant reduction of dimethylnitrosamine-upregulated iNOS and COX2 expression was measured, indicating that kolaviron acts as an antiinflammatory factor. In addition, electrophoretic mobility shift assays showed that this effect may result from reduced formation of the transcription factors nuclear factor "kappa-light-chain-enhancer" of activated B cells (NFκB) and activator protein 1 (AP-1) [29]. Furthermore, interactions of kolaviron with several intracellular immune mediators, such as IL-1α, IL-1β, IL-18, and IL-33, have been observed in murine RAW264.7 macrophages. In this context, kolaviron has been shown to modulate expression and phosphorylation of proteins involved in NFκB, mitogen-activated protein kinase, AP-1, and protein kinase B (PKB/Akt) signaling, leading to an inhibition of the lipopolysaccharides (LPS[1])-induced immune response [30].

Garcinal

The isolation procedure from *G. kola* not only provides garcinoic acid and δ-T3[2], but also garcinal [13-(6-hydroxy-2,8-dimethyl-3,4-dihydro-2H,2--chromenyl)-2,6,10-trimethyl-2,6,10-tridecatrien-1-al] [25]. This structure is closely related to garcinoic acid; solely an aldehyde moiety terminates the side chain instead of a carboxylate moiety (Fig. 9.3).

1. Lipopolysaccharides are endotoxins composed of lipid and polysaccharide components found in gram-negative bacteria that provoke strong immune responses in eukaryotes.
2. Tocotrienols are composed of a chroman ring system and an unsaturated side chain; they constitute a subgroup of vitamin E (the reader is referred to the section "Vitamin E").

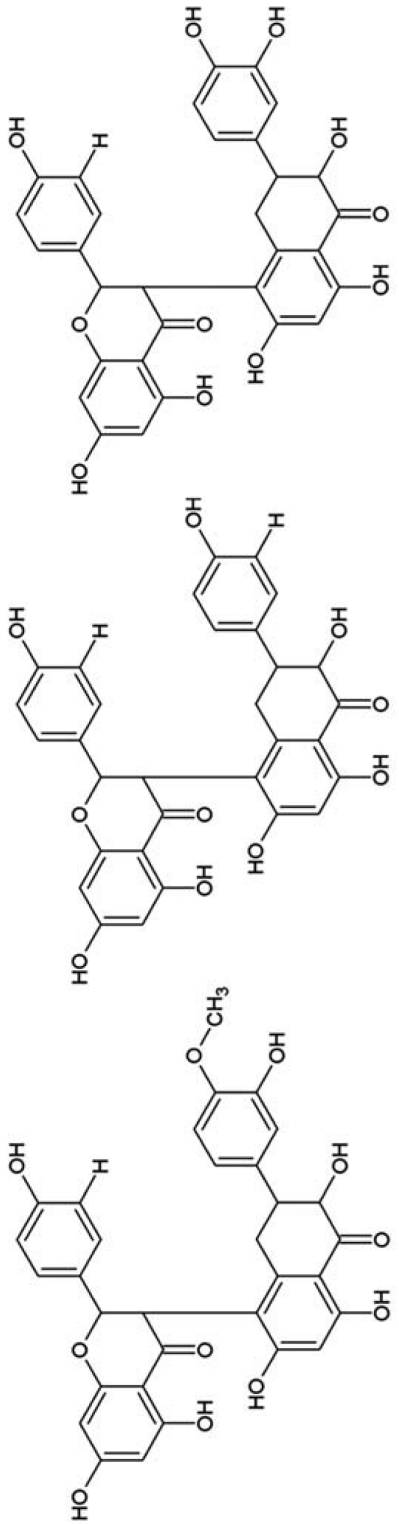

FIGURE 9.2 Chemical structures of biflavones of *Garcinia kola*. *Adapted from O.A. Adaramoye, V.O. Nwaneri, K.C. Anyanwu, E.O. Farombi, G.O. Emerole, Clin. Exp. Pharmacol. Physiol. 32 (2005) 40–46.*

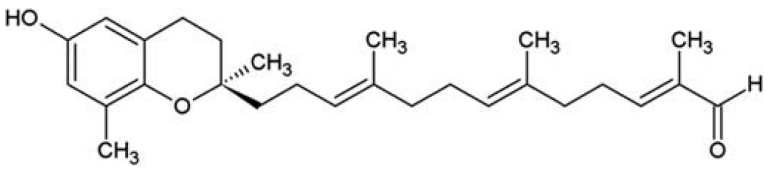

FIGURE 9.3 Chemical structure of garcinal [13-(6-hydroxy-2,8-dimethyl-3,4-dihydro-2H, 2-chromenyl)-2,6,10-trimethyl-2,6,10-tridecatrien-1-al]. *Adapted from K. Terashima, Y. Takaya, M. Niwa, Bioorg. Med. Chem. 10 (2002) 1619–1625.*

However, the metabolic pathways leading to the formation of garcinal in plants have not been elucidated. Based on the structural similarity to garcinoic acid, garcinal likely has a comparable bioactive potential. Nevertheless, the bioactive properties of garcinal are largely unknown. To the best of our knowledge, merely two works addressed the effects of the isolated compound. According to these, garcinal is 1.5 times more potent than α-tocopherol (α-TOH[3]) and has a similar antioxidative activity as garcinoic acid as well as δ-T3 [25]. Furthermore, replacing the terminal functional group of the side chain of garcinoic acid (or garcinal respectively) does not alter the antioxidative capacity [25]. These findings support the hypothesis that garcinoic acid and garcinal may have similar properties in biological systems. Although the health-promoting effects of extracts from *G. kola* (vide supra) are generally ascribed to the biflavones, garcinoic acid and garcinal should be taken into account. This became evident when different fractions of the crude extract were examined regarding their antioxidant and radical-scavenging activities. It turned out that the most potent fraction contained the *Garcinia* biflavone 1 and 2 but also garcinoic acid and garcinal [31]. Given the antioxidative potential of the isolated chromanols, garcinoic acid and garcinal likely contribute substantially to the effects of extracts from *G. kola*. Garcinal is therefore an interesting compound for functional studies due to its structural properties and for explaining the health-promoting effects of *G. kola*.

Garcinoic Acid

Garcinoic acid (*trans*-13′-carboxy-δ-tocotrienol) is an interesting δ-T3 derivative and its occurrence in *G. kola* nuts was first described by Terashima and coworkers in 1997 [32]. A few years later, the same group published a method for the isolation of garcinoic acid from *G. kola* nuts [25]. However, *G. kola* nuts are not the only source of δ-tocotrienolic acid. The extraction of garcinoic acid from members of the *Clusiaceaen* plant family [33] and the development of a stereo-controlled synthesis [34] have been described. For an explicit description of the isolation and synthesis of garcinoic acid, the reader is

3. Tocopherols are characterized by a chroman ring system and a saturated side chain; they constitute a subclass of vitamin E (the reader is referred to the section "Vitamin E").

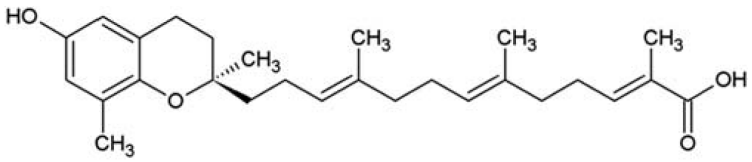

FIGURE 9.4 Chemical structure of garcinoic acid (*trans*-13′-carboxy-δ-tocotrienol). *Adapted from K. Terashima, Y. Takaya, M. Niwa, Bioorg. Med. Chem. 10 (2002) 1619–1625.*

referred to the section "Synthesis of Vitamin E Long-Chain Metabolites." Garcinoic acid is in principle a metabolite of δ-T3 with the carboxylic group placed at the end of the aliphatic side chain (Fig. 9.4), which would be formed in humans in the liver after dietary intake of δ-T3. Thus, garcinoic acid shares structural similarities with δ-T3 [33].

Garcinoic acid shows many bioactive properties. The high antioxidant potential is probably one of the best investigated ones [25,31]. Furthermore, antiproliferative effects were shown in carcinoma cells by Mazzini et al. [33]. The acid also acts as a DNA polymerase β inhibitor, indicating that garcinoic acid is able to disturb base excision repair in tumor cells [34]. This finding supports the results of Mazzini and coworkers. For an explicit description of the bioactive properties of garcinoic acid, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites."

Because of its high content of bioactive components, the *G. kola* nut has great potential for pharmaceutical applications, which is reflected by a number of patents. In 1987, the first patent for a biflavone isolated from *G. kola* as an ingredient for the treatment of liver diseases was registered [35]. The natural product reduced hepatocyte damage in a galactosamine-treated rat model of acute hepatotoxicity and improved liver values in patients with hepatitis [35]. Furthermore, an extract containing a mixture of different biflavones (*Garcinia* biflavones 1, 1a, and 2 as well as kolaflavones) of *G. kola* is used as an antiglycation agent and is also registered in a patent [36]. This compound lowers the accumulation rate of advanced-glycation adducts in the human body; high concentrations of these adducts can damage cells and tissues [36]. The existing patents on bioactive compounds of *G. kola* for the use as pharmaceuticals provide evidence for the growing interest in this plant [35,36]. The role of *G. kola* as an important part of the African ethnomedicine evolved to an interesting source of natural compounds for modern drug development. Although only patents on biflavones have been registered to date, garcinoic acid is also a promising lead compound for future pharmaceuticals.

VITAMIN E

Vitamin E is naturally found in a variety of plant products, such as oils, nuts, germs, seeds, and in smaller quantities in vegetables and some fruits. Due to their lipophilic character, the several molecules summarized as "vitamin E"

are associated to fats in dietary sources. In fact, vitamin E is a hypernym for different molecules, which can be classified as TOH, T3, and a less consistent group of vitamin E–related structures (Fig. 9.5). The common feature of all molecules is the chroman ring and a covalently connected phytyl-like side chain, whose respective constitutions define the individual vitamin E forms. Characteristic for the TOH is their saturated side chain, whereas T3 carry three double bonds in this substructure. The methylation pattern of the chroman ring determines the classification as α-, β-, γ-, or δ-form of the TOH or T3, respectively. More precise, besides position 8, positions 5 and 7 are crucial: α means methylation at position 5, 7, and 8, β at position 5 and 8, γ at position 7 and 8, and δ solely at position 8 of the chroman ring. Natural forms of vitamin E exist in the RRR configuration (TOH) or the R configuration (T3), whereas synthetic vitamin E is a mixture of the different stereoisomers. Members of the group of the vitamin E–related structures can either be more similar to TOH, such as tocomonoenol or marine-derived TOH, or to T3, such as desmethyl-(P_{21})T3, desmethyl-(P_{25})T3, and plastochromanol-8 (Fig. 9.5).

Biological Significance of Vitamin E

Although it is controversially discussed how vitamin E benefits human health, it is an essential factor, as the classification as a vitamin shows. Vitamin E was discovered in 1922 as vital for the fertility of rats [36a], but is also essential for the maintenance of human health. Several disease states have been linked to vitamin E deficiency. A severe effect of inadequate vitamin E supply is anemia. Vitamin E is known for its strong antioxidative properties; if these are lost, erythrocytes are prone to rupture due to higher fragility of their cell membrane [37]. Based on this observation, erythrocyte hemolysis was used as a biomarker to set the recommended daily allowance of 15 mg per day for adults [37]. Not only erythrocytes, but also components of the nervous system are negatively affected by vitamin E deficiency. An isolated vitamin E deficiency, i.e., a deficiency not caused by fat malabsorption, characterizes "ataxia with vitamin E deficiency."[4] This disease is caused by defects in the gene encoding for the α-TOH transfer protein, namely *TTPA*, leading to an impaired ability to retain α-TOH and to depleted α-TOH plasma levels [38,39]. Likely due to the loss of antioxidant protection, nerve cells degenerate and neurological symptoms such as ataxia, dysarthria, hyporeflexia, and decreased vibration sense occur [40].

Vitamin E deficiency might also occur due to fat malabsorption, for example, caused by cystic fibrosis or some liver diseases as well as genetic

4. Ataxia with vitamin E deficiency is an autosomal recessive disorder characterized by markedly reduced plasma levels of vitamin E, ataxia (neurological symptom with a lack of voluntary coordination of muscle movements), spinocerebellar degeneration, and peripheral neuropathy that resembles Friedreich ataxia.

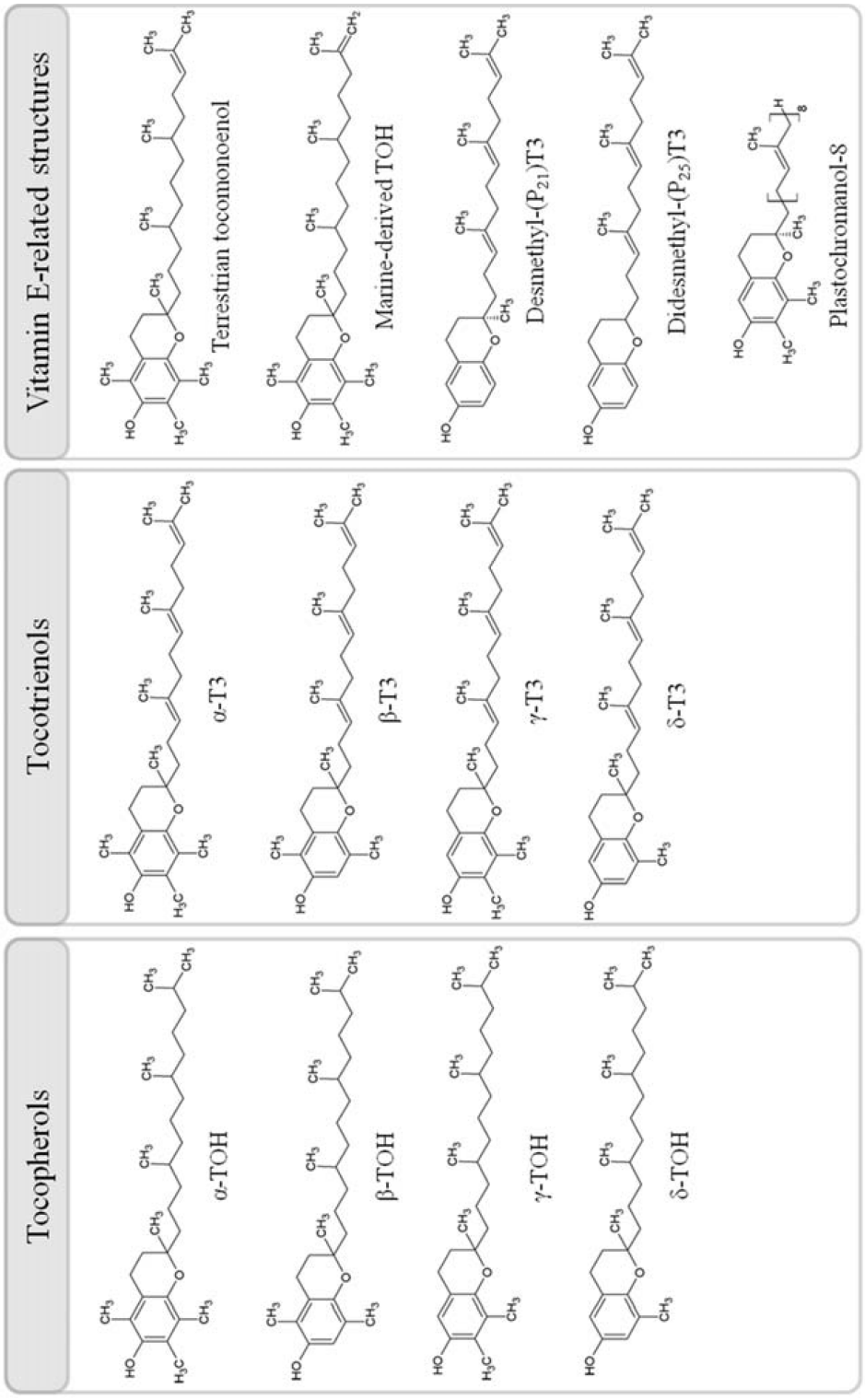

FIGURE 9.5 Chemical structures of vitamin E forms and vitamin E–related natural compounds.

defects, such as abetalipoproteinemia [41–43]. Further, the Marinesco-Sjögren syndrome and chylomicron retention disease likely cause vitamin E deficiency, as they are characterized by impaired chylomicron assembly or delivery [44,45]. Consequently, peripheral nerves die due to the lack of vitamin E, leading to spinocerebellar ataxia. Long-term vitamin E deficiency is further characterized by muscle degeneration. This process can ultimately lead to death if the heart muscle is affected [46]. Given its protective role on neurons, vitamin E was expected to prevent age-related neurodegenerative diseases such as Alzheimer disease. Indeed, vitamin E supplementation slowed down the progression of Alzheimer disease in some human intervention trials [47,48]. Supportive findings were also made in mice, where vitamin E deficiency caused axonal degeneration in brain areas important for memory and cognition [49]. Furthermore, impaired motor coordination and cognitive function was normalized by supplementation with vitamin E in vitamin E–depleted mice [50].

Vitamin E status seems to be important not only for the maintenance of neurons, but also for their development. Several animal studies suggest that the sufficient supply with vitamin E (of the mother) is critical for the development of the central nervous system and cognitive function of the offspring [51–53]. Furthermore, vitamin E along with folic acid may play a supportive role in the prevention of neural tube defects in human [54,55].

For a long time, the effects of vitamin E were attributed to its antioxidant properties (vide supra), but more recent work was dedicated to its non-antioxidant properties. Hence, it became evident that vitamin E modulates gene expression and enzyme activities and interferes with signaling cascades independent of its antioxidative capacity [56]. Examples for such functions are the suppression of inflammatory mediators, reactive oxygen species (ROS[5]), and adhesion molecules; the induction of scavenger receptor; and the activation of NFκB [57]. Given these (and further known) actions, vitamin E is most likely playing a role in several, but not only, inflammatory diseases (for more details, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites"). In addition, T3—another relevant form of vitamin E in our diet—are gaining more attention. Neuroprotective, anticancerogenic, antidiabetic, and cardioprotective effects have been suggested for this group of vitamin E [58]. However, further research is required, as the results obtained from clinical trials for TOH are inconsistent with respect to beneficial effects on chronic diseases such as cancer and cardiovascular diseases (CVD[6]) [59].

5. Reactive oxygen species are oxygen-containing molecules that are highly reactive, such as superoxides, peroxides, hydroxyl radicals, and singlet oxygen.
6. Cardiovascular diseases comprise disorders of the heart and blood vessels including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, pulmonary embolism, and others.

Absorption, Transport, and Distribution of Vitamin E

Vitamin E comprises a class of lipophilic molecules and hence its intestinal uptake follows the pathway known for lipids. A key step is the lipid emulsification, i.e., the incorporation into micelles formed with the help of phospholipids and bile acids. The transfer into enterocytes of the intestine is carried out by passive diffusion, scavenger receptor class B type 1 (SRB1) [60], or Niemann-Pick C1-like protein 1 [61]. As there are no specific transport plasma proteins known for α-TOH [62], it is assumed that vitamin E transport in blood follows that of lipoproteins (reviewed in Ref. [61]). Here, key players in the uptake of vitamin E are SRB1 in peripheral tissue and low-density lipoprotein (LDL) receptor as well as LDL receptor–related protein in the liver [63,64]. Once in the liver, discrimination between the different forms of vitamin E occurs. Responsible for this process is the α-tocopherol transport protein (α-TTP), which promotes the incorporation of *2R*- or *RRR*-α-TOH into very-low-density lipoproteins (VLDL) [65,66], whereas other forms and stereoisomers are secreted into bile [67]. Besides α-TTP, the TOH-associated protein and the TOH-binding protein are known mediators of the intracellular transport of vitamin E. Interestingly, α-TOH secretion from the liver is apparently not dependent on VLDL assembly and secretion, thus oxysterol-binding proteins [68] and ATP-binding cassette transporter A1 (ABCA1) [69] have been suggested to contribute to the release from the liver. Furthermore, ABCA1 mediates the efflux of vitamin E in the intestine, macrophages, and fibroblasts [69], and multidrug resistance P glycoprotein has been identified as a transporter for the excretion of α-TOH via bile [70].

Metabolism of Vitamin E

The metabolism of vitamin E mainly takes place in the liver, whereas extrahepatic pathways have also been suggested [71,72]. Interestingly, rates of vitamin E metabolism increase with higher levels of the vitamin to prevent its accumulation to toxic levels. As indicated before, the preferred form of vitamin E in humans is α-TOH, which is due to the preferential binding of a specific hepatic protein, namely α-TTP. It has been hypothesized that α-TTP protects the α-form from metabolism, in turn leading to its enrichment. Given the lower affinities of the other vitamin E forms to α-TTP, their rate of catabolism is likely more pronounced [73]. In principle, metabolism of all forms of vitamin E follows the same route, which was confirmed by the detection of the respective end products of hepatic metabolism, α-, γ-, and δ-carboxyethyl-hydroxychromanol (CEHC) (Fig. 9.6) [74,75]. However, the catabolic rates depend on the vitamin E form, possibly due to different affinities to key enzymes [73,76]. The classification of the metabolic end product as α-, γ-, and δ-CEHC indicates that the chroman ring is not modified in this process; the aliphatic side chain is rather the substructure

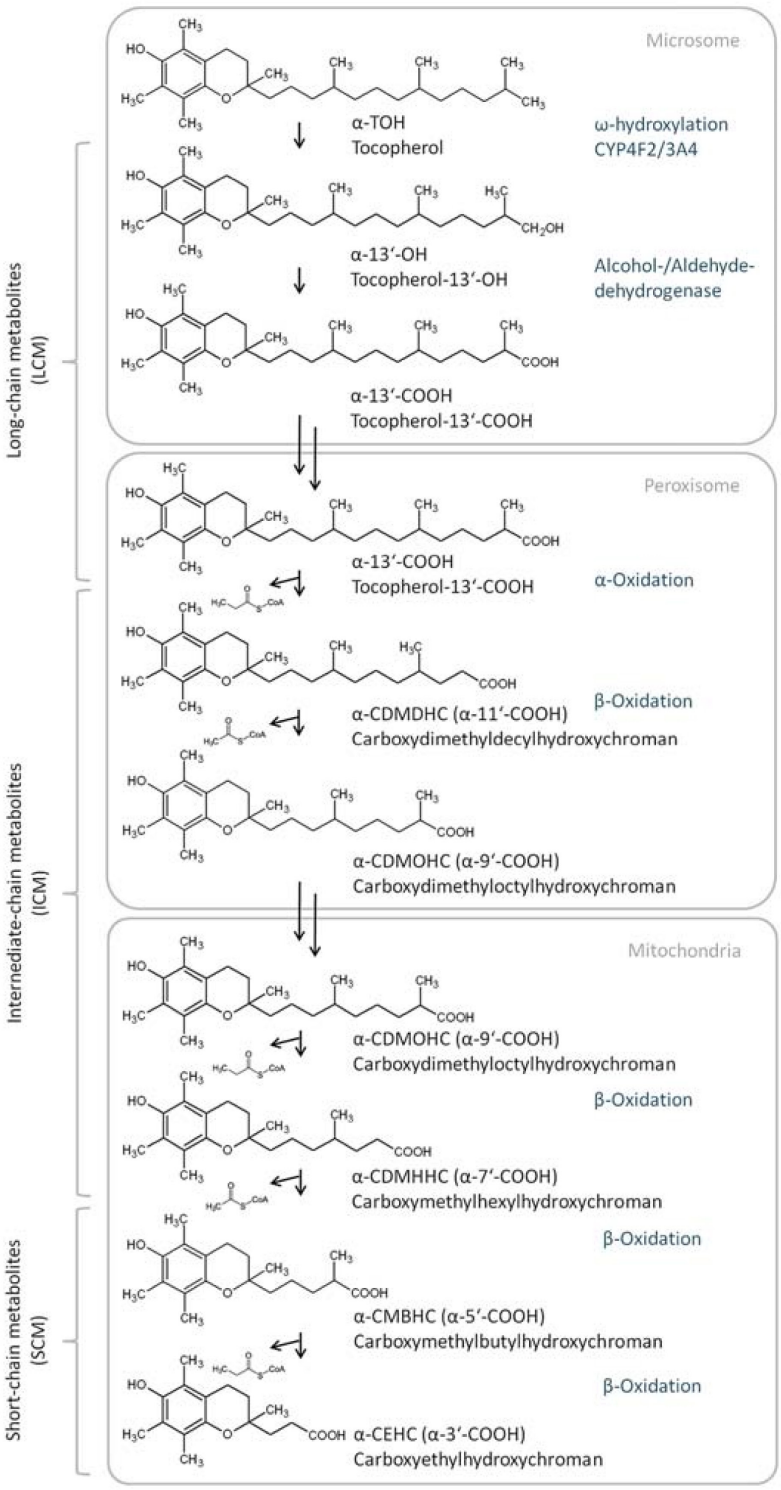

FIGURE 9.6 Principle hepatic metabolism of vitamin E. *Adapted from M. Birringer, P. Pfluger, D. Kluth, N. Landes, R. Brigelius-Flohé, J. Nutr. 132 (2002) 3113–3118.*

where modification takes place. The same applies to T3, whereas further enzymes such as 2,4-dienoyl-coenzyme A (CoA) reductase and 3,2-enoyl-CoA isomerase (known from the metabolism of linoleic acid) are likely needed for metabolizing the unsaturated side chain [77].

Metabolism of vitamin E is therefore characterized by the shortening of the side chain *au fond*. Catabolism of the vitamin E molecule takes place in three cell compartments: the endoplasmic reticulum (microsomes), peroxisomes, and mitochondria. However, the transfer of the metabolites between the compartments is not yet understood. The initial step takes place at the endoplasmic reticulum and results in the formation of 13′-hydroxychromanol (13′-OH) metabolites via ω-hydroxylation by cytochrome P450 (CYP) 4F2 or CYP3A4, respectively [76,78]. Subsequent ω-oxidation by alcohol and aldehyde dehydrogenase (an aldehyde intermediate is formed) leads to 13′-COOH metabolites. Hence, the metabolites are handled like fatty acids and the side chain is shortened by β-oxidation, resulting in the elimination of propionyl-CoA or acetyl-CoA, respectively. The first two rounds take place in the peroxisome, leading to the intermediate-chain metabolites 11′-COOH and 9′-COOH, respectively. Three further rounds of β-oxidation are carried out in the mitochondria, forming the short-chain metabolites (SCM) 7′-COOH and 5′-COOH as well as the final product CEHC or 3′-COOH. During catabolism, the metabolites are modified simultaneously by conjugation, i.e., the metabolites are either sulfated or glucuronidated, but glycine-, glycine-glucuronide-, and taurine-modified metabolites have also been identified [79]. The more hydrophilic conjugated SCM are released via urine. In human urine, however, vitamin E is mainly found in conjugated form after glucuronidation [75,80–82]. The long-chain metabolites (LCM[7]) and their metabolic precursors are secreted via bile into the intestine. This fecal route is considered as the major way of excretion for vitamin E. In contrast to urine, the metabolites in fecal samples are not conjugated [80,83].

SYNTHESIS OF VITAMIN E LONG-CHAIN METABOLITES

The LCM can be obtained in vitro by incubation of cultured cells with the respective TOH precursors (the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites"). The culture supernatants of these cells can be used to investigate the cellular effects of the LCM or their action on isolated enzymes, as it has been already practiced by Jiang et al. [84]. However, this method is not feasible for all investigations, as the cells produce a mixture of carboxychromanols with different chain lengths, including SCM, as well as sulfated and nonconjugated metabolites. Furthermore, not all cell types exhibit the capability to metabolize all forms of TOH

7. The long-chain metabolites of vitamin E are the metabolites of tocopherols and tocotrienols with a side chain that is comprised of 13 carbon atoms.

[85,86]. A purification of defined metabolites is therefore needed if one is interested in investigating the specific effects of a single metabolite.

An alternative way to obtain pure metabolites is their chemical (semi) synthesis. The semisynthesis of α- and δ-13′-OH and the respective 13′-COOH metabolites has been established using the natural product garcinoic acid [33,87]. The first step in the entire process is the extraction (or synthesis) of garcinoic acid from appropriate sources, which is described in the following section. The subsequent synthesis of the α- and δ-LCM from garcinoic acid is outlined in another section.

Isolation of Garcinoic Acid

The isolation of garcinoic acid was first mentioned in 1984 by Franco Delle Monache and colleagues, who used *Clusia grandiflora* from Venezuela as source material [88]. In general, the family of *Clusiaceae* is the source of choice for isolating garcinoic acid. The *Clusiaceae* family is comprised of about 40 genera including about 1600 species, which are found in tropical regions worldwide [89–91]. Members of the family are sources of, inter alia, edible fruits, drugs, pigments, and dyes [90] and have therefore been used in traditional medicine in the regions of their occurrence [89]. So far, three genera of the *Clusiaceae* are known to contain garcinoic acid, namely *Tovomitopsis*, *Clusia*, and *Garcinia*. An overview of reported isolation procedures is provided in Table 9.1.

Tovomitopsis psychotriifolia, a plant from Costa Rica, has been shown to contain garcinoic acid in its leaves. In 1995, Setzer et al. extracted the compound from fresh chopped leaves using 80% aqueous ethanol with a subsequent isolation by liquid chromatography and thin-layer chromatography (TLC) using a 1:1 ethyl acetate/hexane mixture. Determination of the structure was carried out by nuclear magnetic resonance (NMR). Here, the detected structure was *trans*-δ-tocotrienolic acid, whereas Monache et al. mainly found the *cis*-isomer [92].

Among the *Clusia* genus, several members produce garcinoic acid. The trunk of Brazilian *Clusia obdeltifolia* contains a mixture of garcinoic acid in its *cis*- and *trans*-configuration. Extraction of the compounds from dried and powdered material was carried out by hexane with subsequent evaporation of the solvent [93]. Following fractionation with ethyl acetate/hexane and hexane/acetone on a silica column led to the isolation of garcinoic acid. Here, the *cis*-form was more prominent than the *trans*-form with an approximate ratio of 9 to 1, as determined by NMR [93]. The related plant *Clusia burlemarxii*, found in Brazil, also contains garcinoic acid in its leaves. The natural product was extracted from the dried and powdered material by maceration with 95% ethanol, concentration, mixing with 80% ethanol and subsequent treatment with ethyl acetate. Garcinoic acid was then purified by column chromatography over silica gel with mixtures of

TABLE 9.1 Overview of Procedures for Garcinoic Acid Isolation

		Method[a]					
Plant	**Source**	**Extraction**	**Separation Process**		**Input**	**Yield**	**Refs.**
Tovomitopsis psychotriifolia	Leaves	EtOH	LC, TLC	HEX/AcOH	0.16% of starting weight		[92]
Clusia obdeltifolia	Trunk	HEX	CC	**1.** EtAc/HEX **2.** HEX/ACE	6 kg	1.512 g	[93]
Clusia burlemarxii	Leaves	**1.** EtOH **2.** EtAc	CC	**1.** TCM/MeOH **2.** AcOH/MeOH	1.6 kg	5 mg	[89]
Clusia pernambucensis	Bark	EtAc	CC, TLC	**1.** cHEX/EtAc **2.** EtAc/MeOH	197 g	85.3 mg	[94]
			HPLC	H_2O/MeOH/ACN			
Garcinia kola	Seed	**1.** MeOH **2.** MeOH/TCM	CC	**1.** MeOH/TCM **2.** HEX/ACE	1 kg	3.8 g	[87]
Garcinia amplexicaulis	Bark	**1.** DCM **2.** MeOH	CPT	HEP/EtAc/ MeOH/H_2O	270 g	10 mg	[95]

ACE, acetone; *ACN*, acetonitrile; *AcOH*, acetate; *CC*, column chromatography; *cHEX*, cyclohexane; *CPT*, centrifugal partition chromatography; *DCM*, dichloromethane; *EtAc*, ethyl acetate; *EtOH*, ethanol; *HEP*, heptane; *HEX*, hexane; *HPLC*, high-performance liquid chromatography; *LC*, liquid chromatography; *MeOH*, methanol; *TCM*, chloroform; *TLC*, thin-layer chromatography.
[a]*For detailed information, the reader is referred to the text.*

chloroform and methanol in increasing polarity and in a second washing step with mixtures of ethyl acetate and methanol in increasing polarity. Again, the *cis*-isomer was more prominent [89]. A third member of the family, *Clusia pernambucensis* from Brazil, contains garcinoic acid in the bark [94]. The extract was obtained by maceration with ethyl acetate and subsequently fractionated by column chromatography with a cyclohexane/ethyl acetate gradient and sequentially an ethyl acetate/methanol gradient. After profiling with TLC, the appropriate fraction was purified by reverse-phase high-performance liquid chromatography (HPLC) using an isocratic 8:32:60 mixture of water, methanol, and acetonitrile. In addition to the *cis*-isomer of garcinoic acid, the related compounds δ-T3, δ-T3 alcohol, and δ-T3 methyl ester were obtained. However, in terms of quantity, garcinoic acid was substantially more abundant than the other compounds [94].

Members of the genus *Garcinia* are another valuable source of garcinoic acid. The isolation of garcinoic acid from seeds of *G. kola*, which originate from Nigeria, was first described by Terashima et al. in 1997 [25,96]. Based on this procedure, Birringer et al. developed a modified method [87]. Here, the mashed seeds were extracted with methanol, and after evaporation of the solvent, the extract was dissolved in a 95:5 mixture of methanol and chloroform. The crude extract was obtained by drying. For the isolation of garcinoic acid, the extract was again dissolved in 95:5 methanol/chloroform and applied to a silica gel column for purification. Further chromatographic separation on silica gel with a 65:35 mixture of hexane and acetone led to purified garcinoic acid, as characterized by NMR and mass spectroscopy (MS) [87]. A further member, *Garcinia amplexicaulis* from New Caledonia, contains garcinoic acid in the bark. Extraction of garcinoic acid from dried and grounded material was carried out with dichloromethane and subsequently methanol in a Soxhlet apparatus. The extract was further fractionated with a 2:1:2:1 mixture of heptane, ethyl acetate, methanol, and water using centrifugal partition chromatography. Garcinoic acid was subsequently isolated from the appropriate fraction by preparative HPLC using methanol and determined by NMR and MS [95].

Synthesis of Garcinoic Acid

With the first isolation and description of garcinoic acid (δ-*trans*-tocotrienolic acid) from *Clusia grandiflora*, the groundwork for approaches to synthesize this bioactive compound was laid. In 2005, David Maloney and Sidney Hecht reported a procedure to synthesize garcinoic acid (Fig. 9.7).

The basis for their stereo-controlled synthesis was to elaborately produce two molecules: alkyl iodide, (*S*)-1-iodo-5-(2,5-dimethoxy-3-methylphenyl)-3-methylpentan-3-ol (**4**), and vinyl iodide, (2*E*,6*E*,10*E*)-ethyl 11-iodo-2,6,10-trimethylundeca-2,6,10-trienoate (**5**). The alkyl iodide (**4**) was synthesized in two reaction steps from 4-(2,5-dimethoxy-3-methylphenyl)

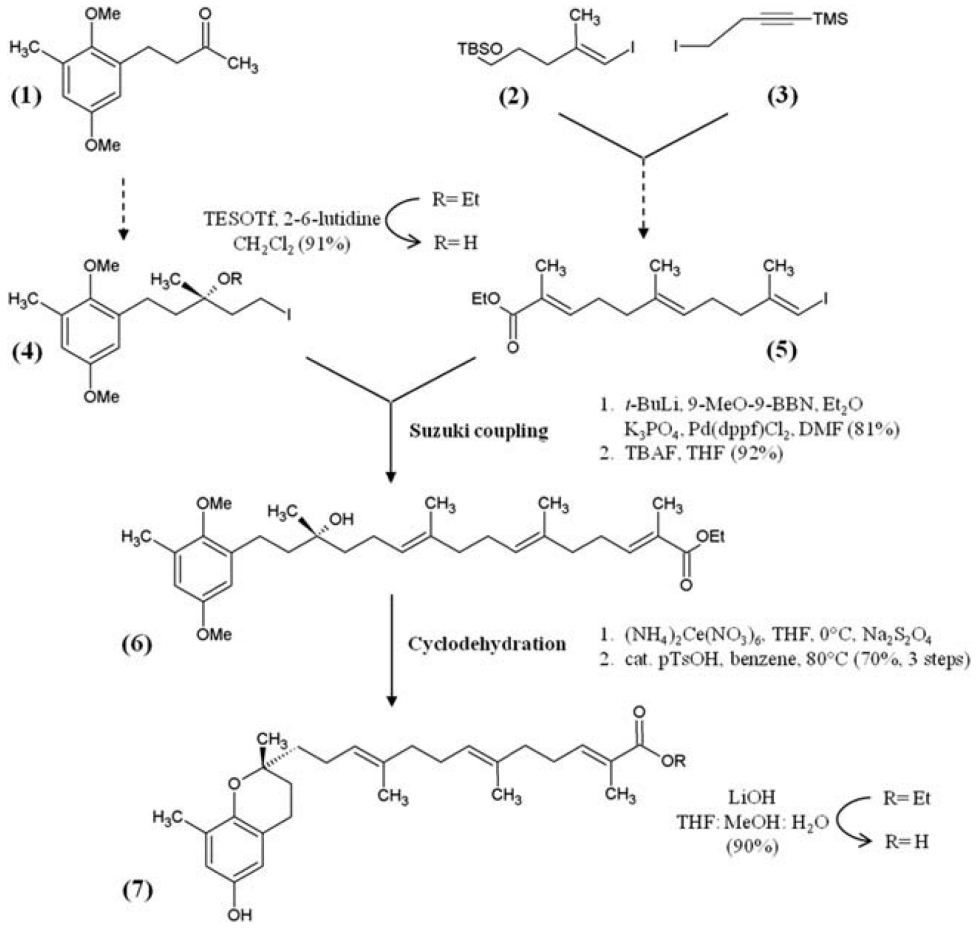

FIGURE 9.7 Stereo-controlled synthesis of garcinoic acid. *Adapted from D.J. Maloney, S.M. Hecht, Org. Lett. 7 (2005) 4297–4300.*

butan-2-one (**1**). The Negishi coupling of *tert*-butylsilyloxy-5-iodo-4-methylpent-4-ene (**2**) and 4-iodo-1-(trimethylsilyl)but-1-yne (**3**) yielded the vinyl iodide (**5**). Suzuki coupling of (**4**) and (**5**) gave the protected prenylated 1,4-benzoquinone, (2*E*,6*E*,10*E*,14*R*)-ethyl 14-hydroxy-16-(2,5-dimethoxy-3-methylphenyl)-2,6,10,14-tetramethylhexadeca-2,6,10-trienoate (**6**). The acid-catalyzed cyclodehydration followed by saponification leads to synthetic garcinoic acid (**7**) [34]. In principle, this synthesis route provides an alternative way to obtain the α- and δ-LCM, starting with synthetic garcinoic acid.

Semisynthesis of Long-Chain Metabolites From Garcinoic Acid

Garcinoic acid, either isolated from the various natural sources or chemically synthesized, can be used for the semisynthesis of α- and δ-LCM. Mazzini et al. reported the respective approach in 2009 [33]. A synthesis route leading to α-TOH was outlined, using the isolated compound from *G. kola* (obtained according to the procedure provided by Terashima et al. [25]) (Fig. 9.8).

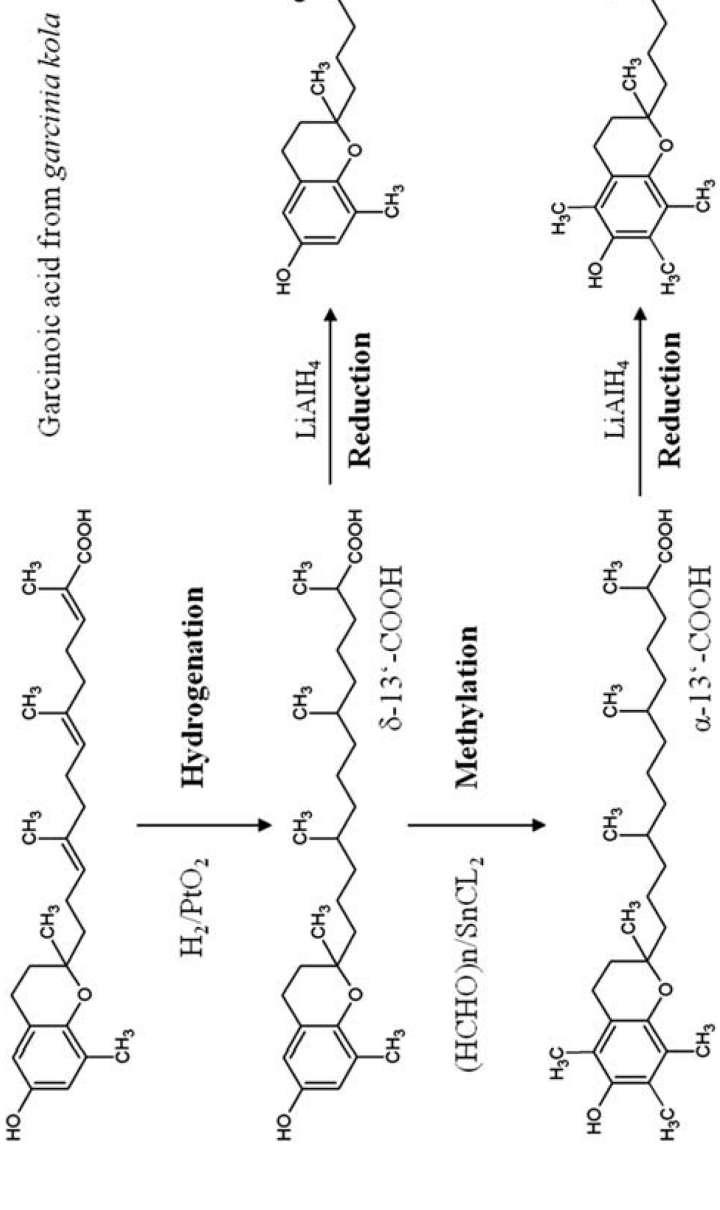

FIGURE 9.8 Semisynthesis of α-13'- and δ-13'-LCM of vitamin E from garcinoic acid. *Adapted from M. Birringer, D. Lington, S. Vertuani, S. Manfredini, D. Scharlau, M. Glei, M. Ristow, Free Radic. Biol. Med. 49 (2010) 1315–1322.*

Here, the unsaturated side chain of garcinoic acid is first hydrogenated in a platinum-catalyzed reaction to receive δ-13′-COOH. The corresponding α-LCM, α-13′-COOH, is obtained by permethylation of δ-13′-COOH, catalyzed by $SnCl_2$. A reduction with $LiAlH_4$ leads to α-13′-OH. To obtain α-TOH, the alcohol can be converted into a ditosylate derivative, and subsequently, the tosyl groups are removed by treatment with $LiAlH_4$ and heating in an aqueous basic solution (not shown). Finally, a hydroxy group resides at the chroman ring and the chain loses its functional moiety [33]. This synthesis route was reproduced by Birringer et al. later. Again, *G. kola*—derived garcinoic acid was used, but δ-13′-OH was derived by reduction of δ-13′-COOH with $LiAlH_4$, additionally. Hereby, the δ-LCM as well as the α-LCM can be obtained from garcinoic acid at sufficient purity for further usage in functional assays [87].

BIOACTIVITY OF GARCINOIC ACID, VITAMIN E, AND LONG-CHAIN METABOLITES

Several functions of vitamin E have been proposed until today. In the early days of vitamin E research, the focus was on the radical chain breaking and radical scavenging capacity of α-TOH, which is regarded as the most potent member of the vitamin E family in this respect [97]. However, Angelo Azzi was the first who provided evidence for further properties of α-TOH that are independent of its function as an antioxidant. He found that α-TOH regulates several cell functions via modulation of signal transduction, nuclear receptors, as well as gene and protein expression besides its function as a natural antioxidant [98,99]. T3 possess similar and sometimes even stronger biological activities than TOH; in particular, T3 show antioxidative, antiatherogenic, anticancer, antidiabetic, antiinflammatory, and neuroprotective properties [58,100]. Apart from the well-known functions of the different vitamin E forms, the bioactivity of their metabolites is not well understood.

Vitamin E metabolism has been studied intensively since the 1990s, but it took about a decade until the first groups were able to detect α-, γ-, and δ-13′-OH as well as the corresponding 13′-COOH metabolites in cell culture supernatants [76], in human liver cells [87], and also in human serum [101]. Current research on LCM is focused on their antiinflammatory properties. Investigations of different groups showed regulatory actions of the LCM on enzymes of the inflammatory cascade [102,103]. Further studies revealed antioxidative and cytotoxic effects [33,87], as well as regulatory properties in lipid metabolism [101]. Based on these studies, the LCM seem to have higher activity and modes of action different from those of the respective vitamin E forms. Garcinoic acid is a natural compound with high structural similarity to the LCM of δ-T3 (and identical to the 13-carbon side chain acid metabolite) [25], indicating that the bioactivity of this substance may be comparable to the LCM of TOH and T3. However, only a few studies on the biological actions of

garcinoic acid have been described so far. The acid exhibits high antioxidative potential [25,31] and antiproliferative effects [33]. However, almost nothing is known about its antiinflammatory or regulatory potential.

The following paragraphs provide an overview on the properties of garcinoic acid and the different LCM in comparison to their precursors.

Cytotoxicity

Recent animal studies on toxic effects of natural or nonnatural vitamin E forms and derivatives on reproduction and development revealed no toxic effects [104]. Physiological vitamin E intake can be increased up to 300 mg/day (mixture of TOH and T3, ~190 IU/day) without causing any complications [105,106]. No clear adverse effects have been described, even for short-term high-dose administration of vitamin E. However, persistent high-dose supplementation has been shown to interfere with blood clotting and is therewith associated to an increased risk of hemorrhagic stroke in animal studies [104]. In the past, TOH was considered to be a safe food additive [107], but an increase in total mortality after high-dose vitamin E intake was discussed during the last years [108]. However, excessive intake of vitamin E results in increased metabolite formation and excretion [109]. This could be a hint that the metabolites of vitamin E may cause noxious effects after a high-dose intake of vitamin E.

Cytotoxic Effects of Vitamin E

Reports on cytotoxic effects of vitamin E are inconsistent. There are considerable differences in the cytotoxicity of the different vitamin E forms. McCormick and coworkers investigated the cytotoxic potential of α-, γ-, and δ-TOH in RAW264.7 macrophages. Concentrations up to 60 μM γ-TOH and especially δ-TOH decreased cell viability by 50% and 90%, respectively, whereas α-TOH had no effect [110]. This has been confirmed in CEM/VLB100 and murine C6 glioma cells [111,112]. Experiments with δ-TOH in different cell types, such as MCF-7 cells, HepG2 cells, and fibroblasts, indicate that δ-TOH-triggered cytotoxicity may depend on the cell type. While δ-TOH incubation results in a massive reduction of viability in MCF-7 breast cancer cells and fibroblasts, no effect was observed for HepG2 liver cells [110]. The first hypothesis—the cell type—dependent cytotoxicity due to different intracellular accumulation of TOH—was disproved [110]. Another concept implies that the degree of methylation of the chroman ring is important for cytotoxicity [110].

In comparison to TOH, T3 show diverse cytotoxic effects. In A549 and U87MG cells, δ-T3 exhibited the highest cytotoxicity followed by γ- and α-T3. Further, the cytotoxicity of T3 derivatives also depends on the cell type [113]. Moreover, cell viability was also reduced in HepG2 liver cells by 40 μM of δ-T3 or γ-T3 [114]. Thus, T3 are able to reduce cell viability in cell types where TOH have no effect. Taken together, the δ-forms of TOH and T3 seem to be the most

cytotoxic vitamin E forms. Moreover, lower concentrations of T3 are needed compared to TOH. TOH and T3 are also known to affect cell proliferation. Antiproliferative effects of all TOH forms have been observed in C6 glioma cells with concentrations higher than 50 μM. Here, α-TOH and γ-TOH were the most potent proliferation inhibitors [112]. The underlying mechanism is probably a block of the cell cycle via p27-mediated inhibition of the cyclin E/cyclin-dependent kinase 2 complex [115] and by increased p53 expression [116]. In particular γ-TOH and δ-TOH, but not α-TOH, affect these pathways [112].

Similar effects can be induced by T3. Because of their higher reactivity, antiproliferative effects of T3 have been studied in cancer cells to use T3 as therapeutic reagents. T3—δ-T3 more effectively than γ-T3—reduced cell proliferation in HL-60, A549, and U87MG cells by induction of apoptosis [113,117]. Thus, TOH (cell cycle arrest) and T3 (apoptosis) exert their anti-proliferative effects via different mechanisms.

Metabolites of Vitamin E

As mentioned before, high doses of vitamin E increase formation of metabolites and their excretion. Therefore, TOH and T3 metabolites might contribute to cytotoxic effects of vitamin E. Studies of Conte et al. in 2004 provided first impressions of CEHC-mediated cytotoxic effects in cancer cell lines. In this work, γ-TOH, γ-T3, and γ-CEHC inhibition of cell proliferation were compared to their respective α-homologues. It should be emphasized that the γ-forms of TOH and T3 have higher transformation rates to CEHC than the respective α-forms. This has been evaluated in PC3, LNCaP, and HepG2 cells [118]. γ-T3 and γ-CEHC are the most potent inhibitors of cancer cell proliferation. At 10 μM, both compounds reduced proliferation of PC3 cells by 70—82%, while their α-analogues were less effective [119]. Francesco Galli and coworkers presume that this effect is triggered by a block of cyclin D1, but further investigations are needed to prove this concept [119]. In conclusion, the SCM are as effective as their precursors in inhibiting cell growth, with γ-forms being most potent.

In contrast to SCM, LCM are widely uncharted. Based on earlier results of Galli et al. and Conte et al. indicating that carboxy-SCM exhibit pro-apoptotic properties, Birringer et al. discovered similar effects for the 13′-LCM [87,118,119]. In this study, HepG2 cells were incubated with α-13′-COOH and δ-13′-COOH and α-13′-OH and δ-13′-OH. The carboxy metabolites appeared to be potent inducers of cell death, while the hydroxy metabolites did not affect cell survival. Furthermore, the δ-forms have been more active than the α-forms. This is reflected by the EC_{50} values of the two substances: 6.5 μM for δ-13′-COOH and 13.5 μM for α-13′-COOH [87], in comparison to α-TOH ($EC_{50} > 100$ μM) and α-CEHC, which showed very low antiproliferative effects at concentrations >10 μM [119]. This finding is in line with the observation that α-13′-COOH and δ-13′-COOH significantly increased the

ratio of apoptosis of HepG2 cells, compared to their metabolic precursors α-TOH and δ-TOH [87]. The treatment of HepG2 cells with α-13′-COOH and δ-13′-COOH also caused increased expression of caspase-3, which is a key enzyme of apoptosis. While δ-13′-OH slightly increased caspase-3 expression, α-13′-OH, α-TOH, and δ-TOH did not [87].

To sum up, the LCM show effects on cell proliferation and cell viability similar to those of their metabolic precursors, but there are significant differences in their activity and the LCM act at much lower concentrations.

Garcinoic Acid

Based on its structural similarities to δ-13′-COOH, it is hypothesized that garcinoic acid has comparable antiproliferative and cytotoxic properties as other vitamin E analogues. To confirm this hypothesis and to get more information about the structural requirements for antiproliferative properties, Mazzini et al. [33] investigated cell proliferation in glioma C6 cells after incubation with garcinoic acid. The acid reduced growth of C6 cells by 50% at concentrations of 10 μM. This effect has also been observed for α-CEHC and δ-CEHC in this study, indicating that the length of the side chain has barely influence on the antiproliferative properties [33,119]. Nevertheless, δ-13′-COOH and α-13′-OH showed higher inhibitory effects on proliferation of C6 cells than α- and δ-CEHC. This indicates that the presence of the carboxyl or hydroxyl group of the vitamin E metabolites enhances antiproliferative effects [33,87]. Based on the limited data on the cytotoxicity of garcinoic acid, its properties seem to be comparable to the other vitamin E metabolites. We found that garcinoic acid showed cytotoxic effects in the RAW264.7 mouse macrophage model system in which we revealed EC_{50} concentrations of about 5.5 μM (unpublished data).

The cytotoxicity of natural compounds is of particular interest for cancer treatment. Several plant-derived anticancer agents are already in clinical use. In particular, taxanes, camptothecines, vinca alkaloids, and podophyllotoxins are worth mentioning [120]. The compounds exert different modes of action, but all have been shown to have antiproliferative effects on cancer cells [121–124]. This is also a characteristic of garcinoic acid, making it interesting for cancer research. Although the effects of garcinoic acid on cancer cells and the underlying mechanisms have still to be characterized, one promising property is already known: garcinoic acid inhibits DNA polymerase β with an IC_{50} of about 4 μM [34]. Compared to other natural DNA polymerase β inhibitors, garcinoic acid is one of the most potent ones (reviewed in Ref. [125]). Cells deficient in DNA polymerase β activity are hypersensitive to certain chemotherapeutic agents due to their impaired ability to repair induced DNA damage [126]. For this reason, the further characterization of the cytotoxic effects of garcinoic acid is of great interest. If garcinoic acid is able to induce DNA damage and simultaneously to suppress DNA damage repair mechanisms, it might be a

powerful agent for cancer treatment. However, the effects of garcinoic acid should first be well characterized in cellular systems before experiments in animal models or even clinical trials in humans can be conducted.

Antioxidative Properties

The antioxidative properties of the different vitamin E forms and metabolites have been extensively studied during the last decades, considering α-TOH as the most important antioxidant, mainly due to the protection against peroxidation of polyunsaturated fatty acids (PUFA[8]) in phospholipids of cellular membranes and plasma lipoproteins, a finding made at least in vitro [56,127]. Higher PUFA intake requires higher vitamin E supply to provide adequate antioxidative protection against lipid peroxidation. Unsaturated fatty acids tend to form radicals, which can be scavenged by the free hydroxyl group at the chroman ring of α-TOH; the reaction product is afterward excreted to bile as α-TOH hydroquinone [128]. All TOH and T3 forms exhibit antioxidative properties. Besides the free hydroxyl group, the mobility of the molecule in cellular membranes is a crucial factor [97,129]. The T3 have higher membrane mobility due to their unsaturated side chain. This should lead to an increase in their antioxidative capacity compared to the respective TOH forms. Yoshida and coworkers compared the effects of either TOH or T3 treatment on peroxyl radical scavenging, but no differences were detectable in membrane uptake or reactivity. However, another investigation on leptosome complexes revealed different results. In this experiment, α-T3 and α-TOH were integrated separately into synthetic membranes. Afterward, lipid peroxidation was induced in another part of the liposomal complex. It appeared that α-T3 was more potent in inhibiting peroxyl radical formation than its TOH equivalent. The more pronounced antioxidative potential of α-T3 seemed to be a result of its better intermembrane mobility, making α-T3 able to reach the radicals faster than α-TOH [130]. This observation has been confirmed by Serbinova and coworkers in rat liver microsomes [97]. However, there are also studies showing similar antioxidant activities of TOH and T3 [130,131].

In addition to membrane mobility, the number of methyl groups of the chroman ring increases the antioxidative capacity of TOH and T3. Despite this, the position of the methyl group in relation to the hydroxyl group at the chroman ring is important. For this reason, the α-forms have higher antioxidative potential than β-, γ-, and δ-derivatives. This has been shown for TOH and T3 in liposomal membranes. After induction of peroxyl radical–triggered lipid peroxidation, the α-derivatives were the most potent inhibitors of oxidative stress. The antioxidant activity decreased from α

8. Polyunsaturated fatty acids are a class of fatty acids characterized by more than one double bond; they are often essential for human nutrition.

through β to γ down to δ [130]. A further investigation in rat serum confirmed this observation [132]. Apart from these results, there are several in vitro studies indicating a reverse order of antioxidant efficiency with α-TOH being the least potent compound compared to δ- and γ-TOH [133,134]. In conclusion, TOH and T3 are highly potent antioxidants with a theoretically decreasing antioxidant activity from α- through β- to γ- and down to δ-forms. Furthermore, T3 seem to be more active than the respective TOH equivalents.

Due to the similarity of the chemical structure of garcinoic acid with T3, comparable antioxidant activities of these compounds can be expected. The antioxidative properties of this natural compound have been investigated in two independent studies. Okoko and coworkers used a methanolic extract from *G. kola* seeds for in vitro experiments. First, the extract was divided into five fractions by TLC. Afterward, the radical scavenging abilities of each fraction were compared to those of vitamin C. The fraction with the highest activity in hydroxyl radical scavenging was further investigated via HPLC analysis. Chromatographic fractioning and spectroscopic analysis revealed four compounds, including *Garcinia* biflavones GB1 and GB2, garcinal, and garcinoic acid [31]. The combination of these four compounds had a 40% higher antioxidative activity than vitamin C at a concentration of 0.5 μg/mL. Further investigations in U937 macrophage cells revealed inhibitory effects on nitric oxide formation [31]. However, Okoko and coworkers were not able to draw a conclusion whether a single compound or the combination of the four substances is responsible for the observed effects. The lack of compound-specific investigations is a crucial limitation of this study. In another investigation, the antioxidative potential of garcinoic acid has been compared to α-TOH using antioxidant activity assays. Terashima et al. found that the antioxidant activity of the natural product was 1.53 times that of α-TOH. This value was comparable to δ-T3 (1.47) and δ-TOH (1.53), molecules sharing high structural similarity to garcinoic acid [25]. Terashima and coworkers chemically modified garcinoic acid by shortening of the side chain. It appeared that the antioxidative activity was significantly affected by structural features, i.e., the shorter the side chain the higher the antioxidative potential. The garcinoic acid analogue with the shortest side chain had 18.7 times higher antioxidant activity than α-TOH [25]. To conclude, garcinoic acid seems to be one of the most potent antioxidative compounds in *G. kola* seeds with an antioxidant activity comparable to compounds such as δ-TOH and δ-T3.

The lack of in vivo studies with garcinoic acid makes predictions difficult whether the antioxidative capacity of garcinoic acid can contribute to drug development and disease treatment. Natural antioxidants in general are believed to have beneficial effects on different diseases. One of the best investigated groups of natural antioxidants are the polyphenols. Compounds such as quercetin, resveratrol, and curcumin are well-investigated members of this class of compounds that have almost similar antioxidative properties as garcinoic acid. All three substances are potent radical scavengers, especially

for hydroxyl radicals [135–137]. Furthermore, quercetin and curcumin have inhibitory effects on nitric oxide formation in different cell types [138,139]. In contrast to garcinoic acid, the use of these polyphenolic compounds for the treatment of diseases in which oxidative stress is involved has already been investigated in mouse models and humans. For example, natural antioxidants showed beneficial effects in nonalcoholic fatty liver disease (NAFLD[9]) and Alzheimer disease (reviewed in Refs. [140,141]). NAFLD is a metabolic disorder associated with high levels of free fatty acids and an increased cardiovascular and liver-related morbidity [142]. High oxidative and inflammatory damage in hepatocytes can also lead to nonalcoholic steatohepatitis (NASH[10]) [143]. Experiments in mice fed a Western diet showed that quercetin lowers oxidative stress in hepatocytes, which in turn leads to reduced liver steatosis [144]. In addition, resveratrol showed promising effects for NAFLD patients in a controlled clinical trial, mainly through lowering inflammatory markers and the reduction of oxidative stress [145]. Resveratrol was further used in studies on Alzheimer disease. Studies demonstrated the importance of neuroinflammation and oxidative stress in the pathogenesis of this disease. One of the most important factors contributing to the development of Alzheimer disease is β-amyloid, because of its ability to generate superoxide anions and α-carbon-centered radicals. The high ROS production caused by β-amyloid may lead to neuronal death [146,147]. Due to its antioxidant activity, resveratrol was used for the treatment of Alzheimer disease in rats, where the compound protected glioma cells from β-amyloid-triggered oxidative damage [148]. Furthermore, curcumin also protected neuronlike PC12 cells from β-amyloid toxicity and displayed neuroprotective effects larger than those of well-known antioxidants such as α-TOH [149]. Besides studies in cellular models, Lim and coworkers have also shown that dietary curcumin suppresses inflammation and oxidative damage in the brain of Tg2576 mice [150]. Furthermore, the epidemiological study by Ganguli and coworkers provides evidence that the Indian population, known for its curcumin-rich diet, shows reduced prevalence of Alzheimer disease compared to the US population [151].

Based on the fact that oxidative stress is a crucial factor for the development of both diseases and natural antioxidants have already shown promising effects on disease prevention, it can be hypothesized that the antioxidative properties of garcinoic acid bear potential for its use in drug development as well as disease prevention and treatment. The well-known effects of other

9. Nonalcoholic fatty liver disease is characterized by the accumulation of fat in the liver of people with no or low alcohol consumption that can lead to inflammation and scarring of the liver.
10. Nonalcoholic steatohepatitis is hallmarked by the accumulation of fat in the liver of people with no or low alcohol consumption accompanied by chronic inflammation, progressive scarring, and cirrhosis of the liver.

natural antioxidants in the prevention of NASH and Alzheimer disease are a promising starting point for in vivo experiments with garcinoic acid.

Apart from the vitamin E isoforms and garcinoic acid, almost nothing is known about the antioxidant activity of the 13′-LCM. Because of their high reactivity, the two LCM 13′-OH and 13′-COOH may act as prooxidants. To prove this hypothesis, Birringer et al. [87] investigated 13′-LCM-triggered ROS production. HepG2 cells were treated with α-13′-OH, δ-13′-OH, α-13′-COOH, and δ-13′-COOH. The corresponding TOH forms were used as controls. Generation of intracellular and mitochondrial ROS was measured via dichlorofluorescein assay [152]. Incubation with 10 μM α-13′-COOH or δ-13′-COOH increased intracellular ROS formation while α-13′-OH, δ-13′-OH, and both TOH forms showed no effect. Similar effects have been observed for mitochondrial ROS production. Here, α- and δ-13′-COOH increased mitochondrial ROS production by 30—50% while the other compounds had no effect. A decrease in mitochondrial ROS production was observed only for δ-TOH [87]. In conclusion, α-13′-COOH and δ-13′-COOH seem to have strong prooxidant potential while α-13′-OH and δ-13′-OH do not act as prooxidants. Due to the structural similarity to the α-13′-COOH and δ-13′-COOH, it can be expected that garcinoic acid exhibits a similar prooxidant potential, but this has to be confirmed experimentally. These observations differ from the results for the antioxidant effects of the different TOH and T3 forms. Particular attention should be paid to studies showing that α-TOH can possibly act as prooxidant [153,154].

Antiinflammatory Actions

Multiple cell types of the innate immune system and paracrine-acting as well as autocrine-acting mediators contribute to the complex process of inflammation. Here, the interplay of proinflammatory and antiinflammatory mediators is vital for the outcome of the inflammatory process, i.e., resolution or chronic inflammation. CVD and cancer, two of the leading causes of death worldwide, are inflammatory diseases, thus highlighting the importance of research for new antiinflammatory treatment approaches. Moreover, diseases of civilization, such as diabetes and obesity as well as asthma, rheumatoid arthritis, osteoporosis etc., have been linked to inflammation.

For this reason, the natural modulators of inflammation are of particular interest. Although several mediators of inflammation and underlying pathways have been identified, we here draw attention to the factors only, which have been investigated in the context of LCM and garcinoic acid.

Cyclooxygenases and Their Lipid Mediator Products

Eicosanoids comprise a group of lipid mediators involved in inflammation, which include prostaglandins, thromboxanes, leukotrienes (LT) and lipoxins. All eicosanoids are metabolically derived from arachidonic acid. Key enzymes

of the conversion of arachidonic acid to eicosanoids are COX1 and COX2 as well as lipoxygenases (LOX; the reader is referred to the section "Vitamin E and Lipoxygenases"). Arachidonic acid is released by action of phospholipases A_2 from phospholipids of the cell membrane. The bifunctional COX (cyclooxygenation and peroxidation function) forms prostaglandin G_2 from arachidonic acid by cyclization and addition of two molecules of oxygen and reduces it further to prostaglandin H_2. This endoperoxide serves as substrate for specific synthases and isomerases, which form prostaglandins of the E_2, F_2, D_2, and I_2 series as well as thromboxane A_2 [155].

While COX1 is constitutively expressed, COX2 can be induced by a variety of proinflammatory stimuli. Hence, COX2 is regarded as the more important source of eicosanoids during inflammation. All of the abovementioned prostaglandins are implicated in proinflammatory actions (reviewed in Ref. [156]).

Vitamin E Modulates Prostaglandin E_2 Release and Cyclooxygenase Activity

Tocopherol Inhibit Cyclooxygenase Activity

The release of prostaglandin E_2 (PGE_2) is widely used as a marker for the activity of COX. The effect of TOH on the release of PGE_2 has been studied in several cell types and settings. In BV-2 microglia cells the induction of PGE_2 by LPS could be attenuated by α-TOH dose-dependently. While 25 μM showed no effect, 50 μM diminished the effect significantly and 100 μM almost completely blocked the induction [157]. An interesting finding was made in human aortal endothelial cells: α-TOH induced the release of PGE_2 dose-dependently in concentrations above 10 μM. In contrast, COX activity, measured as conversion of exogenous arachidonic acid to PGE_2, was attenuated by α-TOH at 10 μM or higher. The authors postulated that α-TOH induces (1) the release of arachidonic acid from membrane phospholipids and (2) the expression of $cPLA_2$. The discrepancy in the abovementioned results is explained by a more relevant effect of α-TOH on substrate release (i.e., the release of arachidonic acid from membrane phospholipids) than on COX activity [158].

These findings implicate that the effects of TOH on PGE_2 release depend on the cell type. However, similar findings were made in macrophages. In peritoneal macrophages obtained from rats treated with 5 mg/day α-TOH (i.p.) for 6 days, the production of PGE_2 in response to different stimuli was diminished. Interestingly, macrophages from control animals showed a response similar to untreated control cells, when preincubated with α-TOH [159]. In a different approach with peritoneal macrophages the most effective reduction of PGE_2 production was observed with δ-TOH (1.25−12.5 μM) and α-TOH (12.5−150 μM). γ-TOH was less effective and β-TOH had no effect (up to 12.5 μM). Interestingly, all TOH forms reduced COX activity, measured

as the conversion of PGE_2 from exogenous arachidonic acid. Again, δ-TOH was most potent followed by β-, α-, and γ-TOH in descending order [160]. Thus, the substitution of the chroman ring seems to be important for the modulation of PGE_2 synthesis. However, it is possible that the different TOH forms act in different ways, either on substrate availability or on COX activity.

Are Tocotrienols the More Potent Vitamin E Form?

T3 have also been shown to be potent inhibitors of PGE_2 release. In malign mammary epithelial cells, PGE_2 release was reduced about 50% of controls by 3 μM γ-T3 [161]. Different effects were observed in mouse RAW264.7 macrophages stimulated with LPS to induce PGE_2 release and subsequently incubated with three different T3 forms at 10 μg/mL. While γ-T3 showed no effect, δ-T3 was the most potent inhibitor with about 55% reduction followed by a T3-rich fraction and α-T3. Surprisingly, α-TOH increased the effect of LPS induction [162]. In IL-1β-stimulated A549 lung epithelial cells, γ-T3 was as effective as δ-TOH in inhibiting release of PGE_2. The IC_{50} for both compounds were about 1–3 μM. γ-T3 was more potent than its γ-TOH counterpart (IC_{50} of 6–7 μM), while α-T3 exerted only weak inhibitory action (20% at 20 μM), and α- and β-TOH were completely ineffective below 50 μM [84]. The aforementioned results suggest that the T3 are more potent inhibitors of COX activity than their respective TOH forms. However, the substitution pattern of the chroman ring appears to be also a major determinant for the effectivity of the compound.

Tocopherol Metabolites Outclass Their Metabolic Precursors

While little is known about the bioactivity of TOH LCM in general, some studies focused on their effects on COX activity. We recently reported that α-13′-COOH is a potent COX-regulating metabolite. In mouse RAW264.7 macrophages, the upregulation of COX2 mRNA and protein by LPS and the subsequent increase in PGE_2 release was diminished by α-13′-COOH and α-TOH. Whereas α-TOH reduced PGE_2 production about 55%, α-13′-COOH abolished PGE_2 production almost completely. These findings are of particular significance as 100 μM α-TOH was less effective than 5 μM of α-13′-COOH. This underlines the higher effectivity of the LCM. In addition to PGE_2, the LPS-induced formation of further arachidonic acid–derived eicosanoids, namely prostaglandin D_2 and prostaglandin $F_{2\alpha}$, was blocked by α-13′-COOH. In contrast, α-TOH did not diminish the induction by LPS significantly [102]. In agreement with this, Jiang et al. reported no effect of 50 μM α-TOH on PGE_2 production in lung epithelial cells [84]. Compared to α-TOH, δ-TOH is more potent in inhibiting PGE_2 production (vide supra). In contrast to Wallert et al., Jiang et al. used no synthetic LCM, but cell culture medium collected from cells treated with TOH, containing the self-synthesized metabolites 9′-COOH, 11′-COOH, and 13′-COOH. An intact-cell assay with preinduced

COX and arachidonic acid as substrate revealed that the medium containing the δ-metabolites is superior to that with γ-metabolites in inhibiting COX activity. Unfortunately, the authors used a cell line that is unable to metabolize α-TOH to its respective carboxychromanols [85,86], resulting in no effect of α-TOH in this assay. δ-9′-COOH and δ-13′-COOH isolated from cell culture supernatants inhibited COX2 with IC_{50} of 6 or 4 μM, respectively. However δ-9′-COOH was unable to inhibit activity of purified COX1 and COX2 enzymes in concentrations <20 μM. In contrast, δ-13′-COOH was highly potent with an apparent IC_{50} of 5 μM for COX1 and 4 μM for COX2, which is comparable to ibuprofen. Only weak inhibition of both COX isoforms was shown for the SCM α-CMBHC (α-5′-COOH; $IC_{50} > 140$ μM) and γ-CEHC (γ-3′-COOH; $IC_{50} > 300$ μM) [84]. This finding indicates that LCM rather than SCM may be responsible for the antiinflammatory effects of TOH. This assumption is supported by the fact that A549 lung epithelial cells are not able to produce SCM [85,86]. Anyway, δ-SCM would be preferable for comparison, as the structure of the chroman ring likely influences the effectivity.

Garcinoic Acid: A New Player on the Court?

To date, no systematic investigation of the modulation of COX activity by garcinoic acid, the principal δ-13′-LCM of δ-T3, has been published. With respect to its structural similarities, garcinoic acid shares the chroman ring with δ-TOH, which has been shown to be the most potent TOH in this context [160]. In addition, δ-T3 is more effective in modulating COX activity than the other T3, which in turn can be considered more effective than TOH [84,162]. The unsaturated chain is a structural feature of garcinoic acid shared with T3. For this reason, we expect that garcinoic acid is more potent in modulating COX than TOH. As garcinoic acid carries a carboxylic acid moiety, one can compare it to the 13′-carboxychromanols generated from TOH. In particular, 13′-COOH have been shown to be substantially more effective in inhibiting COX activity than their metabolic precursors [84,102]. Based on these observations, garcinoic acid is likely more potent than TOH and comparable to (δ-)T3 or its LCM, respectively. However, experiments are required to confirm whether this hypothesis holds true.

Vitamin E and Cyclooxygenase Expression

While the effects of the different vitamin E forms on COX activity are evident, the underlying mechanisms are not yet fully resolved. A common way to decrease the activity of an enzyme—in addition to its inhibition—is its downregulation. As COX1 is constitutively expressed, no regulation is expected nor has been shown experimentally [158,161,162]. Divergent results have been obtained with respect to the influence of vitamin E on COX2 expression. In murine microglia cells, 50 μM α-TOH abolished LPS-induced gene expression and 100 μM moreover reduced protein synthesis of COX2,

likely via NFκB [157]. However, contradictory results were obtained in other studies. COX2 levels were reduced neither by 100 μM α-TOH in LPS-treated murine macrophages [102] nor by 60 μM α-TOH in IL-1β-stimulated human lung epithelial cells [158]. For the suggested molecular mode of action on COX activity, the reader is referred to the section "Tocopherol Inhibit Cyclooxygenase Activity."

In contrast to TOH that may exert posttranscriptional effects on COX activity, T3 have been shown to downregulate COX expression. In LPS-treated RAW264.7 macrophages, 10 μM of α-, γ-, and δ-T3 blocked COX2 expression while α-TOH did not [162]. In line with this, 10 μM of γ-T3 downregulated constitutive COX2 expression in human pancreatic cancer cells and 50 μM completely blocked the expression [163]. These findings are supported by further studies, characterizing γ-T3 [161,164] and δ-T3 [165] as highly efficient suppressors of COX2 expression. Interestingly, in both studies comparing the effects of T3 forms on COX2 expression, δ-T3 was the most potent one [162,165]. The higher ability of δ-T3 to diminish COX2 activity is in accordance with results for the different TOH forms. However, the ability to regulate COX2 expression seems to be a characteristic of T3.

Effect of Long-Chain Metabolites of Vitamin E on Cyclooxygenase 2 Expression

Based on the findings for TOH and T3, it can be assumed that α-TOH LCM are rather ineffective in regulating expression of COX2. Surprisingly, Wallert et al. reported significant blocking of LPS-induced expression of COX2 by α-13′-COOH in murine RAW264.7 macrophages: preincubation with 5 μM α-13′-COOH and subsequent coincubation with LPS significantly diminished the effect of LPS on COX2 expression at mRNA and protein levels. In contrast, 100 μM of α-TOH showed no significant effect [102]. These results show that the α-LCM act in a different fashion and at lower concentrations than their respective metabolic precursors. The underlying pathways have not been elucidated so far and remain to be investigated.

Effect of Garcinoic Acid on Cyclooxygenase 2 Expression

So far, no studies have been published that investigate the effects of garcinoic acid on COX2 expression. Due to the unsaturated chain, garcinoic acid is structurally comparable to δ-T3 but also shares similarities with α-13′-COOH. Considering this, it can be assumed that garcinoic acid may also interfere with the LPS-mediated upregulation of COX2. Preliminary results of our group indicate that garcinoic acid indeed has the potential to block the LPS-induced upregulation of COX2 mRNA as well as protein (unpublished data). However, this is merely a first hint and further experiments are needed. Nevertheless, garcinoic acid would not be the first compound isolated from plants for the treatment of inflammatory diseases in folk medicine. Well-known examples

are curcumin from *Curcuma longa*, capsaicin from *Capsicum* species, and epigallocatechin-3-gallate from *Camellia sinensis* [166]. All of these compounds have been shown to inhibit COX2 expression [167,168]. Especially *C. longa* has been used for centuries in Ayurvedic medicine to treat inter alia the inflammation-related diseases asthma, rheumatism, and diabetes [169]. Today, more than 50 completed clinical trials with curcumin display the interest in this valuable ingredient of *C. longa* in modern medicine. As with *C. longa*, *G. kola* is used in folk medicine to treat inflammation-related diseases (the reader is referred to the section "*Garcinia kola*"). Despite kolaviron, garcinoic acid has now been identified as an antiinflammatory active ingredient of *G. kola*. In principle, garcinoic acid is an interesting natural compound with antiinflammatory actions that should be further characterized. Possibly, the properties of kolaviron and garcinoic acid can be used jointly in the form of a *G. kola* nut extract to treat CVD, cancer, and other diseases of civilization.

Vitamin E and Lipoxygenases

Lipoxygenases and Their Lipid Mediators

LT are formed by LOX, a family of enzymes with four subclasses, namely 5-, 8-, 12-, and 15-LOX, which are classified according to the position at which these enzymes catalyze the dioxygenation of PUFA. The release of arachidonic from membrane phospholipids by $cPLA_2$ is crucial for LT synthesis. 5-LOX catalyzes the oxidation of arachidonic acid and thus the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which in turn is converted to LTA_4 by the same enzyme. LT A_4 is the precursor of LTB_4 or LTC_4, which are in turn the precursors of LTD_4 and LTE_4, respectively, formed by LTA_4 hydrolase and LTC_4 synthase, respectively [170].

Tocopherols Inhibit Lipoxygenase Activity

The first demonstration of 5-LOX inhibition by TOH was published in 1985 [171]. It was shown that α- and γ-TOH inhibit the conversion of arachidonic acid to 5-HPETE by 5-LOX from potato tubers. Interestingly, the inhibition was as efficient as with known 5-LOX inhibitors, such as nordihydroguaiaretic acid and butylated hydroxytoluene, and furthermore irreversible and noncompetitive with arachidonic acid [171]. LT B_4 is a major product of the 5-LOX pathway (vide supra) and is thus widely analyzed in activity and signaling studies. In 1999, Devaraj and Jialal noticed that preincubation of human peripheral blood mononuclear cells (PBMC) with α-TOH (but not β-TOH) impaired the release of IL-1β in response to LPS. Treatment of the cells with LTB_4 restored IL-1β release. By the use of 5-LOX inhibitors it was confirmed that 5-LOX mediates the effects of α-TOH. Furthermore, α-TOH diminished LTB_4 release [172]. A later study of the same group confirmed these results. PBMC isolated from α-TOH-supplemented healthy subjects showed impaired ability to produce TNFα in response to LPS compared to

cells obtained at baseline or after washout. Preincubation of LPS-stimulated PBMC with 50 or 100 μM α-TOH as well as 5-LOX inhibitors showed the same effect. The impaired TNFα release could be restored by LTB_4 [173]. In vitro experiments show that concentrations of 25 μM of α-TOH are not sufficient to inhibit release of TNFα. This raises the question whether a supplementation with 1200 IU/day (corresponds to about 800 mg/day) is sufficient to achieve the required plasma levels of α-TOH. It might be possible that the α-LCM mediate or contribute to the effects observed in vivo, but this remains to be shown experimentally.

Two human trials in hemodialysis patients support the abovementioned findings [174,175]. Patients under hemodialysis exhibit increased 5-LOX levels in their PBMC. In these studies, patients were subjected to α-TOH administration to improve oxidative stress markers [174]. Supplementation with α-TOH, 300 mg/day i.m., 600 mg/day orally [150], or via vitamin E-coated cuprammonium rayon membranes [151] for 4 weeks diminished LTB_4 release and 5-LOX activity. The expression of 5-LOX was not affected by the treatments [175,176].

Although there is evidence that TOH are capable of inhibiting 5-LOX activity and LTB_4 production, further research is required. The majority of studies were done on α-TOH, but the different vitamin E forms seem to act differently and there might be more potent forms [171–173]. Furthermore, α-TOH was administered in the mentioned human trials and the effects were attributed to the TOH itself, regardless of metabolic conversions.

Effects of Metabolites and Tocotrienol on Lipoxygenase Activity

Despite the observation that T3 inhibit 12-LOX activity (reviewed in Ref. [177]), little is known about T3 and their effects on LOX. In fact, just a single study addressed effects of T3 on 5-LOX. In this study, γ-T3 was compared to different TOH forms and δ-13′-COOH [103]. For this, HL60 cells were differentiated into neutrophils and eosinophils to induce 5-LOX expression. Activity of 5-LOX was subsequently stimulated by different concentrations of the calcium ionophore A23187 and measured as formation of LTB_4 and LTC_4. In cells incubated with 1 μM, α-TOH was less effective (IC_{50} = 60 and 40 μM, respectively) than its γ- and δ-counterparts (IC_{50} = 5 μM). Interestingly, γ-T3 was as effective as γ-TOH, and δ-13′-COOH was the most potent compound tested (IC_{50} = 4 μM). Strikingly, δ-13′-COOH inhibited formation of LTB_4 with an apparent IC_{50} of 7 μM, when cells were stimulated with 5 μM A23187, while none of the other vitamin E forms was able to inhibit 5-LOX activity in this setting with concentrations up to 50 μM. The superiority of δ-13′-COOH was confirmed in a cell-free assay with recombinant 5-LOX. Here, the LCM efficiently inhibited the activity of 5-LOX with an IC_{50} of 0.5–1 μM, while all the other vitamin E forms failed to inhibit 5-LOX with concentrations of up to 50 μM. The efficiency of the carboxychromanol is thus similar to that of zileuton, a specific inhibitor of the 5-LOX-activating protein. For this

reason, δ-13′-COOH is thought to inhibit 5-LOX directly. However, final evidence is pending. An alternative way by which δ-13′-COOH may modulate 5-LOX activity is by inhibiting the increase in intracellular Ca^{2+} levels in response to N-formylmethionine leucyl-phenylalanine or thapsigargin. In this respect, the metabolite was superior to its metabolic precursor δ-TOH, which failed to inhibit the induction in calcium influx [103].

Garcinoic Acid and Lipoxygenase Activity

No experimental data regarding garcinoic acid and LOX activity are currently available, but based on the observation that the structurally related LCM δ-13′-COOH is a potent inhibitor of 5-LOX activity, garcinoic acid may likely exert similar effects on this enzyme. The finding that γ-T3 is also able to inhibit 5-LOX activity with an efficiency comparable to γ-TOH further supports this hypothesis, because the unsaturated chain has obviously no effect on the inhibitory capacity. The same may likely be true for garcinoic acid, but this has to be confirmed experimentally. 5-LOX and its products are involved in many inflammation-related diseases, including CVD, cancer, osteoporosis, inflammatory bowel disease, rheumatoid arthritis, skin diseases, and bronchial asthma. The latter is the major 5-LOX-associated disease and zileuton, the only approved 5-LOX inhibitor so far, is available for treatment [178]. Nevertheless, zileuton has two major drawbacks, liver toxicity and a short half-life [179]. There is thus an urgent need to find new potent 5-LOX inhibitors. Many natural products have been identified as 5-LOX inhibitors (reviewed in Ref. [178]). However, most of them are not well characterized and far from use as drugs [178]. Flavocoxid, a mixture of the bioflavonoids baicalin from *Scutellaria baicalensis* and catechins from *Acacia catechu*, made it to a phase III trial but the problem with this natural 5-LOX inhibitor is the reported risk of acute liver injury [180]. Garcinoic acid could line up with the known natural 5-LOX inhibitors, with the potential advantage of modification of multiple inflammatory pathways simultaneously (the reader is referred to the respective chapters on COX). Furthermore, extracts of *G. kola* have hepatoprotective effects [23], so a nut extract might exert effects on 5-LOX without liver injury. Moreover, garcinoic acid could be hepatoprotective itself, as the related structures TOH and T3 have been reported to be beneficial for liver health repeatedly [181,182]. If garcinoic acid is indeed a 5-LOX inhibitor, its exact mechanism of action should be investigated to assess its clinical potential. The lack of 5-LOX inhibitors with satisfying properties shows the need of new sources for their development and garcinoic acid is a promising candidate.

Modulation of Lipid Homeostasis

In addition to inflammation, dysbalanced lipid homeostasis is a key factor for diseases such as atherosclerosis. A plethora of signaling pathways and cellular

processes are required to regulate lipid homeostasis, involving uptake, intracellular trafficking and storage, metabolism, as well as efflux of lipids. The following sections will only focus on that parts of lipid metabolism that have been linked to the LCM so far, namely, expression of the scavenger receptor cluster of differentiation 36 (CD36), uptake of oxidized LDL (oxLDL), and phagocytosis as well as intracellular lipid storage. These are essential elements of macrophage foam cell formation, which in turn is a key event in the pathogenesis of atherosclerosis.

Tocopherols and Macrophage Foam Cell Formation

Macrophage-derived foam cells contribute significantly to the pathogenesis of atherosclerosis. This cell type is therefore studied extensively with respect to its role in inflammation and lipid metabolism. CD36 is a scavenger receptor that significantly contributes to the uptake of oxLDL and is thus involved in the accumulation of cholesterol in intracellular lipid droplets, a hallmark of macrophage foam cells. Therefore, factors that modulate CD36 expression and the uptake of oxLDL are of particular interest. The ability of TOH to modulate the regulation of CD36 and the uptake of oxLDL as well as subsequent processes has been described in several studies: α-TOH is able to suppress the upregulation of CD36 during macrophage differentiation [183,184]. Furthermore, it blocks the upregulation of CD36 in response to oxLDL in THP-1[11] macrophages [185] and to modified LDL in PBMC-derived macrophages [183]. Moreover, the uptake of oxLDL can be decreased by α-TOH in several macrophage models [183–185]. The incubation with oxLDL causes a lipid accumulation in macrophages, which can be also prevented by α-TOH [185]. In line with this, the accumulation of cholesteryl esters in response to modified LDL is diminished in α-TOH-treated macrophages [183].

The regulatory effects of α-TOH on CD36 have been observed also in mice. Apolipoprotein E-knockout mice fed a diet supplemented with 100 mg/kg α-TOH per day for 8 weeks showed a reduced extent of atherosclerotic lesions as well as the expression of CD36 therein and serum concentrations of oxLDL than the respective control group [186]. Similar findings were obtained in LDL-receptor-knockout mice. Here, supplementation with α-TOH acetate and α-TOH (equivalent to 50 IU vitamin E per kilogram of diet, ad libitum) for 18 months resulted in a decrease in lesional and nonlesional expression of CD36 [187].

These findings are also supported by results from liver disease research. The HepG2 liver cell line shows decreased CD36 expression when treated with α-TOH [188]. Rats fed a diet enriched with 80 IU/kg diet (ad libitum) α-TOH

11. THP-1 cells are a human monocytic cell line derived from an acute monocytic leukemia patient. THP-1 monocytes can be differentiated into macrophages using phorbol 12-myristate 13-acetate.

acetate showed reduced hepatic CD36 mRNA levels compared to controls [189], and a comparable result was obtained with merely 6 mg/kg of the diet α-TOH combined with 11 mg/kg of the diet γ-TOH ad libitum [190]. A study with guinea pigs points to a posttranslational regulatory mechanism of α-TOH decreasing CD36 protein levels in the liver [191].

Effects of Long-Chain Metabolites and Garcinoic Acid on Macrophage Foam Cell Formation

A surprising result was obtained when we examined the effects of LCM on CD36 expression. In contrast to the downregulatory potential of 100 μM α-TOH, its LCM α-13′-OH and α-13′-COOH upregulated CD36 mRNA and protein in human THP-1 macrophages and human PBMC-derived macrophages obtained from healthy volunteers with as little as 10 and 5 μM, respectively. Generally, primary cells showed a slightly lower response. In addition, the increase in CD36 expression by oxLDL was attenuated by α-TOH and markedly augmented with the LCM [101].

Given the LCM-induced CD36 expression, an increase in oxLDL uptake is expected, but this was not the case. Treatment with LCM before addition of oxLDL led to decreased oxLDL uptake in THP-1 and PBMC-derived macrophages. In line with this, the accumulation of neutral lipids by oxLDL was attenuated in LCM-pretreated cells [101]. We found that garcinoic acid also induces the expression of CD36 in the nonproliferating THP-1 macrophage model. Here, the effectivity of garcinoic acid was comparable to that of the α- and δ-LCM (unpublished data).

Since the current state of knowledge on the regulation of lipid metabolism by garcinoic acid is based only on cellular models, it is difficult to draw conclusions whether these observed effects may have an influence on in vivo models. As mentioned before, garcinoic acid shows functions similar to other natural compounds such as resveratrol, especially with regard to its antioxidative properties. For this reason, resveratrol is preferred for deducing possible in vivo effects of garcinoic acid on lipid homeostasis. Independent experiments in THP-1 and 3T3-L1 cells showed that CD36 expression is upregulated by resveratrol [192,193]. Unfortunately the uptake of oxLDL has not been measured in these cell models. In addition to the mentioned in vitro studies, Chen and coworkers investigated the effect of resveratrol treatment on lipid homeostasis in skeletal muscles of rats fed a high-fat diet. After 8 weeks of high-fat feeding, the basal CD36 mRNA expression was increased in the intervention group in comparison to controls. The treatment with resveratrol led to a further induction of CD36 expression [194]. Based on this observation it was quite surprising that the enhanced expression of an important lipid importer did not lead to increased intracellular lipid accumulation, indicating that the induction of CD36 expression by resveratrol has no negative effect on in vivo lipid balance [194]. Because of the similarities between the properties of resveratrol and garcinoic acid, it could be hypothesized that a possible upregulation of CD36 expression by garcinoic acid will also have no negative

effects on lipid metabolism in vivo. However, further experiments are needed to prove this concept.

CONCLUSIONS AND PERSPECTIVES

With the evidence of circulating α-LCM in human blood, a new perspective in vitamin E research was presented. In addition to the well-studied TOH and the latterly more focused T3, their LCM must be taken into account to correctly interpret the effects of vitamin E in humans. We speculate that the LCM comprise a new class of regulatory molecules that complicate the interpretation of studies on the effects of vitamin E in vivo as these molecules exert effects that are different from their metabolic precursors. So far, only a few studies have focused on this class of compounds. However, the LCM seem to share properties with their precursors but to exert also unique or even adverse effects. It is evident that the LCM and precursors act in the same manner with respect to cytotoxicity and modulation of COX2 and 5-LOX activity but it is of note that the LCM are significantly more potent than their precursors in these cases. Hence, the LCM may indeed play a role in mediating these effects of vitamin E in the human body although the blood concentrations are significantly lower than those of TOH. In addition, the LCM exhibit different effects, like their prooxidative capacity reported by Birringer et al. [87]. This in turn is surprising, as vitamin E in general is well known for its antioxidative properties. Moreover, the LCM apparently upregulate CD36, while the downregulation of this receptor by TOH has been shown repeatedly. Furthermore, the LCM can act in areas where the TOH are virtually not effective. A prime example is the regulation of COX2 expression (for more information, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites").

The natural product garcinoic acid is structurally related to the α-LCM. However, little is known about its bioactivity (Fig. 9.9). Merely, its antioxidative and antiproliferative potential as well as its inhibition of DNA polymerase β have been examined. Due to the structural similarities to TOH, T3, and the LCM, many, yet unknown, effects of garcinoic acid can be expected, making garcinoic acid an interesting natural product for pharmacologic research itself. Although little is known on the effects of garcinoic acid, *G. kola* nuts have been reported as inter alia antidotal, antiinflammatory, antidiabetic, and hepatoprotective. It is likely that garcinoic acid contributes to these properties as it has strong antiinflammatory and antioxidative properties. First results support this hypothesis, as garcinoic acid has shown antiinflammatory actions via downregulation of COX2 expression. For this reason it will be interesting to see what effects garcinoic acid shows in different cell and animal models. If the proposed beneficial properties shown in Fig. 9.10 come true, garcinoic acid has to be tested in clinically relevant studies in animals and later on humans. This may lead to the transfer of knowledge from folk medicine to modern medicine to cure disease.

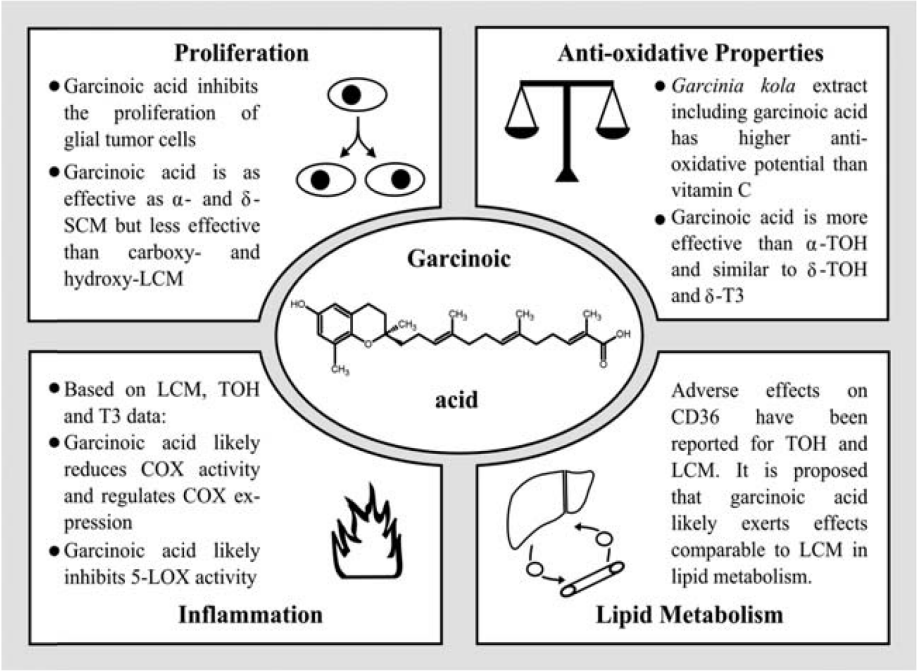

FIGURE 9.9 Known and proposed effects of garcinoic acid.

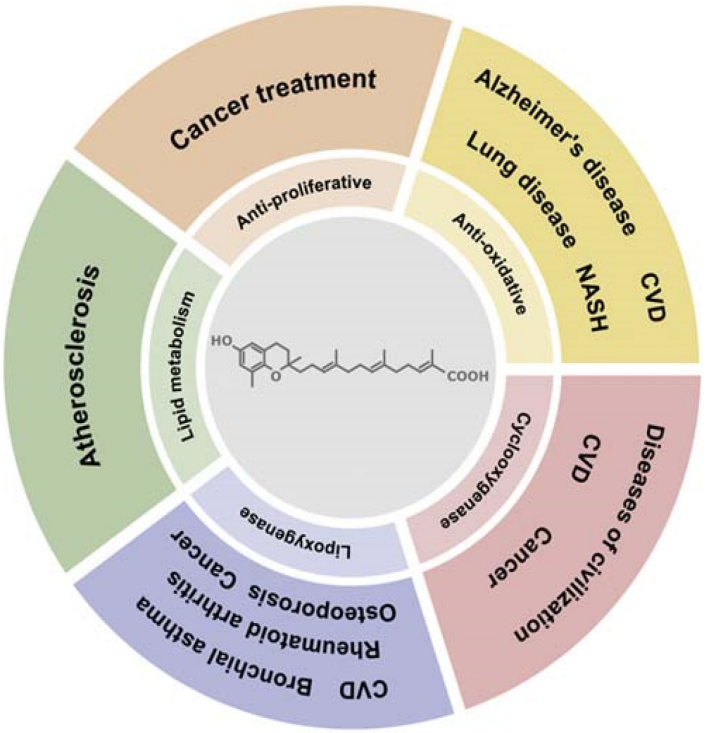

FIGURE 9.10 Proposed beneficial effects of garcinoic acid on human diseases. For detailed information, the reader is referred to the relating chapters. *CVD*, cardiovascular disease; *NASH*, nonalcoholic steatohepatitis.

In addition, garcinoic acid is a helpful substrate that can be reliably isolated from *G. kola* nuts in pure form for synthesizing the δ-LCM, namely δ-13′-OH and δ-13′-COOH, as well as the α-LCM, namely α-13′-OH and α-13′-COOH. The isolation of garcinoic acid from *G. kola* nuts is a simple yet effective method with a yield superior to other reported isolation procedures (the reader is referred to Table 9.1). Thus the procedure provides a reliable base for the synthesis of large amounts of LCM. Pure α- and δ-LCM can be simply and efficiently obtained with the semisynthesis route presented by Mazzini and Birringer. Taken together, this procedure is the most effective way to obtain sufficient amounts of the respective LCM of interest for cellular, animal, as well as human experiments. For this reason, the isolation of garcinoic acid allows the synthesis of the LCM in an elegant and efficient way and the investigation of physiological functions of the α- and δ-LCM in vitro as well as their pharmacological modes of action in vivo in appropriate animal disease models.

To unravel unknown effects and better understand known effects of the different vitamin E forms, as well as to elucidate the underlying regulatory mechanisms that likely involve the LCM, some central questions should be addressed. These include inter alia (1) Which proteins are involved in the uptake and intracellular trafficking of garcinoic acid and of the LCM? (2) Which cellular receptors, signaling proteins, or enzymes mediate the effects of garcinoic acid and of the LCM? (3) What are the regulatory mechanisms that mediate expression of genes in response to garcinoic acid and to the LCM? (4) Which molecular structures are responsible for the effects of garcinoic acid or of the LCM? (5) Do the different LCM differ in their effects and effectiveness? To answer these questions, systematic and comprehensive studies are required. The studies likely involve the identification of potential transporters, binding protein receptors for garcinoic acid, and the LCM. These studies should be complemented by profiling of the effects of garcinoic acid and LCM on gene expression and signaling pathways in different cell types as well as studies in animal models that will shed new light on the regulatory modes of action of the different vitamin E forms and their metabolites. To understand the structure—activity relationship, further structurally related compounds, such as synthetic derivatives of garcinoic acid or of the LCM or enantiomer-pure molecules as well as compounds that represent substructures of the molecule, i.e., the chroman ring or the side chain, should be studied. To sum up, the availability of the LCM as pure compounds provides new perspectives for vitamin E research that will likely contribute to a better understanding of the physiological function of vitamin E. In this respect, the natural product garcinoic acid is a very helpful tool that provides simple and efficient access to the pure α- and δ-LCM for functional studies.

ABBREVIATIONS

13′-COOH 13′-carboxychromanol
13′-OH 13′-hydroxychromanol

5-HPETE	5-hydroperoxyeicosatetraenoic acid
ABCA1	ATP binding cassette transporter A1
ACE	acetone
ACN	acetonitrile
AcOH	acetate
AP-1	activator protein 1
AVED	ataxia with vitamin E deficiency
CC	column chromatography
CD36	cluster of differentiation 36
CEHC	carboxyethyl-hydroxychromanol
cHEX	cyclohexane
CoA	coenzyme A
COX	cyclooxygenase
CPT	centrifugal partition chromatography
CVD	cardiovascular diseases
CYP	cytochrome P450
DCM	dichloromethane
EMSA	electrophoretic mobility shift assays
EtAc	ethyl acetate
HEP	heptane
HEX	hexane
HPLC	high-performance liquid chromatography
ICM	intermediate-chain metabolite(s)
IL	interleukin
iNOS	inducible nitric oxide synthase
LC	liquid chromatography
LCM	long-chain metabolite(s)
LDL	low-density lipoproteins
LOX	lipoxygenase
LPS	lipopolysaccharides
LT	leukotriene
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein 1
MS	mass spectroscopy
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NFκB	nuclear factor “kappa-light-chain-enhancer” of activated B cells
NMR	nuclear magnetic resonance
NPC1L1	Niemann-Pick C1-like protein 1
oxLDL	oxidized LDL
PBMC	peripheral blood mononuclear cells
PGE2	prostaglandin E_2
PKB	protein kinase B (Akt)
PKC	protein kinase C
PUFA	polyunsaturated fatty acid(s)
ROS	reactive oxygen species
SCM	short-chain metabolite(s)
SRB1	scavenger receptor class B type 1

T3	tocotrienol(s)
TCM	chloroform
TLC	thin-layer chromatography
TNFα	tumor necrosis factor α
TOH	tocopherol(s)
TTP	tocopherol transfer protein
VLDL	very-low-density lipoproteins

ACKNOWLEDGMENTS

Work of M.B. is funded by grants from "Forschung für die Praxis" of the Hessisches Ministerium für Wissenschaft und Kunst. Work of S.L. is supported by grants from the Deutsche Forschungsgemeinschaft and the German Federal Ministry of Research and Education as part of the Competence Cluster for Nutrition and Cardiovascular Health (nutriCARD) Halle-Jena-Leipzig.

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7 Diskussion

Die Forschungsaktivitäten rund um die langkettigen Vitamin-E-Metabolite werden bislang nur von einer kleinen Gruppe interessierter Wissenschaftler durchgeführt, obwohl die Aufklärung der biologischen Wirksamkeit der LCM einen erneuten Wendepunkt in der Erforschung des Vitamin E herbeiführen könnte. Für einen schnellen und einfachen Einstieg in die Thematik stehen drei Übersichtsarbeiten (Manuskripte 5 bis 7) zur Verfügung, die die aktuelle Literatur zusammenfassen und die Bedeutung der LCM in den Gesamtforschungskontext einordnen. Um eine kritische Diskussion in der Forschungsgemeinschaft anzuregen, wurden folgende Thesen in die Übersichtsarbeiten aufgenommen, die in den kommenden Jahren bearbeitet bzw. belegt werden sollen.

T1. Die LCM sind eine neue Klasse regulatorischer Metabolite.

T2. Die LCM-vermittelte Regulation verläuft über einen distinkten und spezifischen (von TOH verschiedenen) Mechanismus.

T3. Die LCM interagieren mit physiologischen und pathophysiologischen Prozessen.

T4. Die Diskrepanz, welche im Kontext der kardiovaskulären Forschung zwischen den *In-vitro*- und *In-vivo*-Ergebnissen aufgezeigt wurde, kann durch die biologische Wirkung der LCM erklärt werden.

T5. Die LCM sind als neue Biomarker für die Vitamin-E-Versorgung interessant.

Zur Bestätigung dieser Hypothesen sind unter anderem folgende Fragen experimentell zu beantworten (Auflistung in Manuskript 7):

F1. Welche Proteine sind an der Aufnahme und der intrazellulären Verteilung der LCM und GA beteiligt?

F2. Welche zellulären Rezeptoren, Signalmoleküle oder Enzyme vermitteln die Effekte der LCM und GA?

F3. Welche Mechanismen sind für die Regulation der Genexpression durch die LCM verantwortlich?

F4. Welche molekularen Strukturen sind für die Effekte der GA und LCM verantwortlich?

F5. Unterscheiden sich die LCM in ihren Effekten und in ihrer Effektivität?

7.1 Beitrag der Manuskripte zur Beantwortung der Forschungsfragen

Im Zentrum der vorliegenden Arbeit steht die Aufklärung der biologischen Aktivität der LCM. Dieses weitläufige Gebiet kann im Rahmen einer einzelnen Dissertation nicht vollständig erschlossen werden. Daher knüpft diese Arbeit an die Promotionsschrift von WALLERT (2014) an, beschreibt die eigenen Erkenntnisse und liefert Ansatzpunkte für nachfolgende Arbeiten.

Die gerade formulierten offenen Fragen werden alle in der vorliegenden Arbeit adressiert und

partiell (F2 und F3 in Manuskript 1, 3 und 4) oder auch zum größten Teil beantwortet (F4 und F5 in Manuskript 1). Dabei liegt der Fokus dieser Manuskripte auf den LCM. Während der Entstehungszeit dieser Arbeit wurden unter Mitarbeit der Autorin ebenfalls erste Einblicke in die biologische Aktivität der GA gewonnen (nachfolgend als Projekt 1 bezeichnet), welche aufgrund der hohen Strukturähnlichkeit der LCM zur GA in die Diskussion einfließen sollen. Vorläufige Ergebnisse (Projekt 2) und weitere Konzepte zur Beantwortung von F1 werden vorgestellt. Diese Projekte sollen zeitnah in Veröffentlichungen münden, liegen derzeit aber noch nicht in einreichbarer Form vor. Da die bisherigen Ergebnisse der Projekte in die zusammenfassende Diskussion einfließen sollen, werden sie hier kurz dargestellt.

Das Projekt 1 untersucht die Regulation der Inflammation durch den methanolischen Extrakt der *Garcinia kola* und der enthaltenen GA. Dabei wurde zunächst die Isolation der GA aus *Garcinia kola* optimiert (6,6-fache Isolationseffizienz). In murinen RAW264.7-Makrophagen zeigt die GA konzentrationsabhängig antiinflammatorische Effekte (Hemmung der LPS-induzierten Cox2 und iNos auf mRNA-, Protein-Expression und -Funktion). Apolipoprotein-$E^{-/-}$-*Knockout*-Mäuse wurden mit fettreicher westlicher Diät gefüttert und einmal wöchentlich intraperitoneal für acht Wochen mit 1 mg/ml GA behandelt, um eine während der Atherogenese auftretende Inflammation zu unterdrücken. Dadurch änderte sich die Zusammensetzung, aber nicht die Größe der entstehenden Plaques im Aortenbogen. In diesem Modell spielt eine niedriggradige Inflammation die wesentliche Rolle, während *in vitro* ein hochgradiges Inflammationsmodell abgebildet wird. Daher soll die GA künftig in einem Myokardinfarktmodell (akutes Inflammationsmodell) untersucht werden. Dieses Projekt wird von Maria Wallert in Jena und Australien hauptverantwortlich durchgeführt.

Das Projekt 2 optimiert die Probenaufarbeitung und die Analyse von LCM mittels HPLC-Fluoreszenzdetektion (FD) und LC-MS. Diese Optimierung der LCM-Analytik soll die Messung aller Metabolite und Vorstufen in einem chromatographischen Lauf ermöglichen. Dazu werden Extraktionsmethoden (Flüssig-Flüssig-Extraktion und Festphasenextraktion (Normale SPE (*solid phase extraction*) und Hybrid-SPE) sowie der Einfluss von pH-Wert und enzymatischer Dekonjugation verglichen. Die LC-MS-Analyse der Proben zeigt ebenfalls bislang unidentifizierte Peaks, wie sie schon von GIUSEPPONI *et al.* (2017) beschrieben wurden. Anwendung findet die optimierte Methode in Zellkulturproben und in humanen Serumproben. Dieses Projekt wird in Fulda von Marc Birringer und Alexander Maxones durchgeführt.

Der detaillierte Beitrag der einzelnen Manuskripte zur Beantwortung der Forschungsfragen wird in den nachfolgenden Kapiteln beschrieben. Die Manuskripte 1 bis 4 beschäftigen sich mit der Regulation von zellulären Prozessen durch die LCM. Der Schwerpunkt des Manuskriptes 1 liegt dabei außerdem auf dem Nachweis der engen Strukturabhängigkeit der LCM-Effekte. Auf dieser Basis kann nun die Identifizierung des putativen LCM-Rezeptors angestrebt werden. Damit wird der vermutete distinkte, von TOH verschiedene LCM-vermittelte Mechanismus bekräftigt (T2) und stärkt die Einordung der LCM als regulatorische Metabolite (T1). Abbildung 9 verdeutlicht die Interaktion der Manuskripte und ihren Beitrag zur Beantwortung der Forschungsfragen.

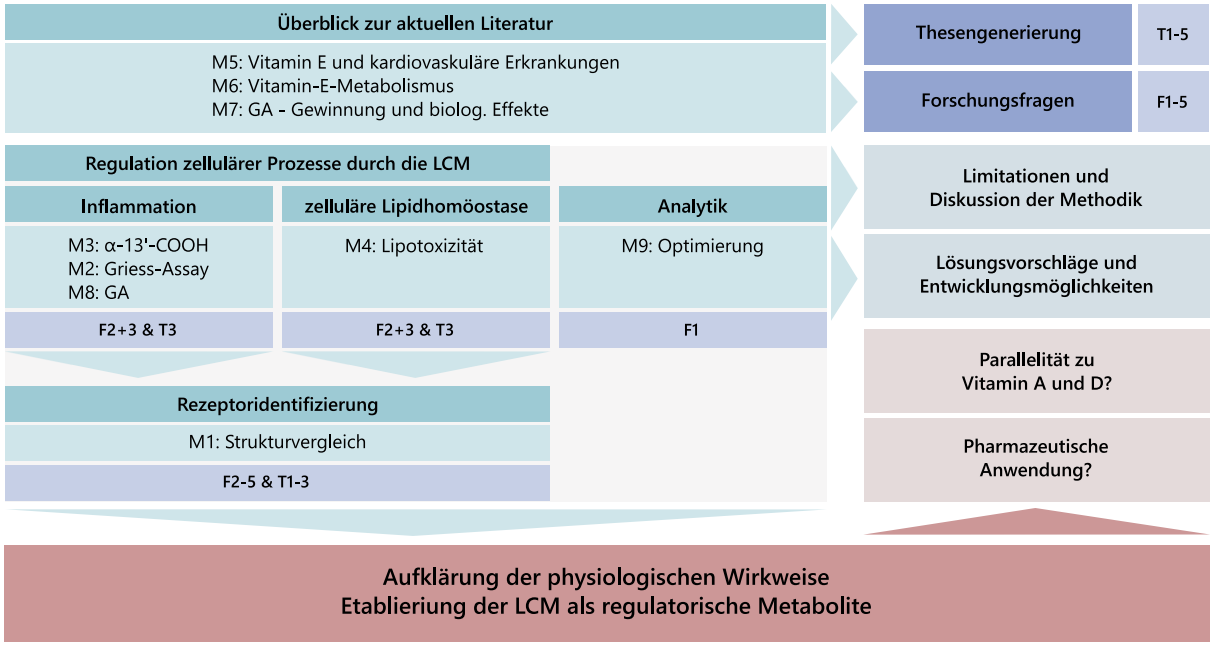

Abbildung 9: Einbettung der Manuskripte in den Forschungskontext.
Die in den Übersichtsarbeiten generierten Forschungsfragen und Thesen werden in den Manuskripten 1 bis 4 und in den Projekten 1 und 2 bearbeitet und bekräftigt. **Verwendete Abkürzungen:** α-13'-COOH, α-13'-Carboxychromanol; F, Forschungsfragen; GA, Garciniasäure; M, Manuskripte; P, Projekte; T, Thesen.

7.1.1 Molekulare Strukturen der LCM

In einer ausführlichen Strukturaktivitätsanalyse werden im Manuskript 1 die biologischen Effekte von α- und δ-TOH, deren LCM (13'-OH und 13'-COOH), sowie α-CEHC und Pristansäure miteinander verglichen. Es zeigt sich, dass die gesamte Struktur der LCM, bestehend aus dem Chromanolring, der Seitenkette und ihrer oxidativen Modifizierung, für die Vermittlung der biologischen Effekte verantwortlich ist (Beantwortung von F4). Damit legen die Ergebnisse des Manuskriptes 1 eine distinkte und spezifische Regulation durch die LCM nahe (T2). Auf dieser Basis kann die Existenz von einem oder mehreren molekularen Rezeptoren der LCM vermutet werden, welche(r) künftig identifiziert werden sollen (F2).

Ausgehend von der gezeigten engen Strukturabhängigkeit der LCM-Effekte könnte für die Rezeptoridentifizierung ein sogenanntes *Fishing*-Experiment vielversprechend sein (siehe Abbildung 10). Dazu werden die LCM immobilisiert und anschließend mit Zelllysaten inkubiert. Proteine können spezifisch an die LCM binden (sog. *protein pull-down assay*). Unspezifische Bindungen werden mit Hilfe von Waschschritten gelöst. Nach der Freisetzung der Proteine werden diese elektrophoretisch im 2D-Gel aufgetrennt. Der Vergleich des LCM-Gels z.B. mit einem Gel, das durch TOH-Immobilisierung entstanden ist, lässt die Isolation spezifischer Proteinspots zu und ermöglicht die Identifikation der Proteine mittels Massenspektrometrie. Auf der Grundlage des Manuskriptes 1 scheint die Immobilisation der LCM über die Hydroxylgruppe des Chromanolrings erfolgversprechend.

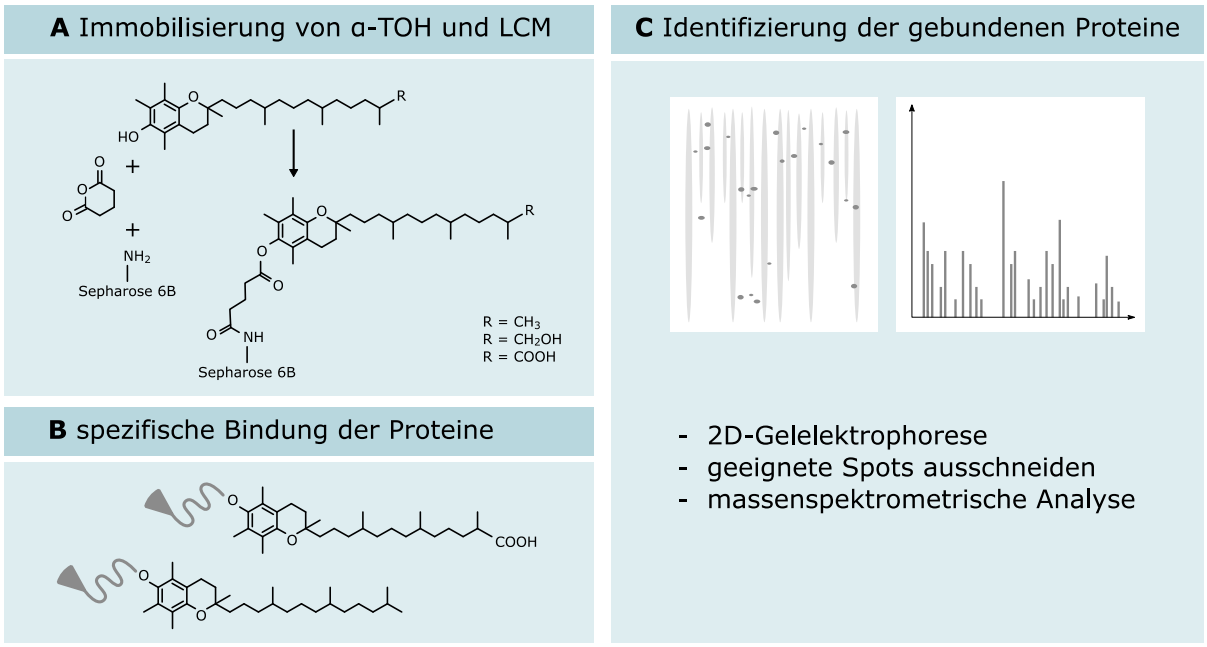

Abbildung 10: Rezeptoridentifizierung über *Target-fishing*-Experimente (siehe Text für Erläuterungen).
Verwendete Abkürzungen: α-TOH, α-Tocopherol; LCM, *long-chain metabolites*.

7.1.2 Biologische Effektivität der LCM

Das Manuskript 1 beschreibt außerdem offensichtliche Effektivitätsunterschiede der LCM und beantwortet damit F5. Beinahe unabhängig von der Substitution des Chromanolringsystems zeigen die untersuchten LCM konsistent einheitliche Regulationen. Die Effektgröße hingegen hängt von der Modifikation der Seitenkette ab. So sind Effekte der 13'-COOH-Metabolite jeweils in geringerer Konzentration (5 µM) größer als die der 13'-OH-Metabolite (10 µM). Die LCM besitzen also in Abhängigkeit von ihrer spezifischen Struktur eine unterschiedliche biologische Effektivität. Folgende Aspekte können zu diesem Phänomen beitragen.

Unterschiede in der zellulären Bioverfügbarkeit der Substanzen sind als Ursache denkbar. Daher werden zur Aufklärung der Aufnahmeeffizienz (siehe Kapitel 7.3.8 und Beantwortung von F1) Zellen mit den Metaboliten inkubiert (THP-1- und RAW264.7-Makrophagen mit α-LCM) und anschließend analysiert (Projekt 2). Dabei zeigt sich, dass die Zellen α-13'-COOH effektiver aufnehmen (THP-1: ~ 4 µM und RAW264.7: ~ 11 µM intrazelluläre Konzentration bei 5 µM Inkubationskonzentration) als α-13'-OH (THP-1: ~ 5 µM und RAW264.7: ~ 8 µM intrazelluläre Konzentration bei 10 µM Inkubationskonzentration). Möglicherweise erklärt die geringere intrazelluläre Konzentration von α-13'-OH in den RAW264.7-Zellen die schwächeren Effekte.

Unerwartet ist der folgende Befund: In den Proben, die mit α-13'-OH inkubiert werden, kann intrazellulär α-13'-COOH gemessen werden (THP-1: ~ 3 µM und RAW264.7: ~ 6 µM). Dies deutet auf einen intrazellulären Abbau von 13'-OH zu 13'-COOH hin. Es ist denkbar, dass das neugebildete α-13'-COOH die eigentlich effektive Substanz darstellt. Die durch α-13'-OH vermittelten Effekte könnten zum einen deshalb geringer ausfallen, weil die intrazelluläre Konzentration des neugebildeten α-13'-COOH geringer ist, als bei einer direkten Inkubation mit α-13'-COOH. Zum andern muss der Metabolismus der Vermittlung der Effekte zunächst vorausgehen (Zeiteffekt). Da über die Kinetik des LCM-Metabolismus wenig bekannt ist (siehe

Kapitel 7.3.5), kann über die Größe dieses Einflussfaktors nur spekuliert werden. Basierend auf den gerade vorgestellten Daten kann die Hypothese aufgestellt werden, dass 13'-OH evtl. erst nach einer Bioaktivierung effektiv ist.

Angenommen, ein oder mehrere spezifische LCM-Rezeptoren existieren, so kann spekuliert werden, dass 13'-COOH durch die räumliche Struktur die Bindungstasche des putativen Rezeptors besser ausfüllt und damit effektiver ist als 13'-OH. Zur Bestätigung dieser Idee werden zunächst vielversprechende Kandidaten identifiziert (Rezeptor-*Fishing*). Eine anschließende Kristallisation des Rezeptors mit den LCM könnte die endgültige Bestätigung liefern.

7.1.3 Regulation zellulärer Prozesse durch LCM

Die Regulation der zellulären Lipidhomöostase wurde und wird auf den drei Ebenen Lipidaufnahme über CD36 (WALLERT *et al.* 2014a und Manuskript 1), Lipidspeicherung über PLIN2 (Manuskript 4) und Lipidexport über ABCA1 (laufendes Projekt, siehe Kapitel 7.4.1) untersucht. Gemeinsam lassen die Ergebnisse ein umfangreiches Wirkbild der LCM entstehen.

Im Manuskript 1 wird die Induktion von CD36 auf mRNA- und Proteinebene durch α- und δ-LCM beschrieben. Diese Daten bestätigen die Ergebnisse von WALLERT *et al.* (2014a) und erweitern sie um die Informationen für die δ-LCM. Damit ist ein formübergreifender Vergleich der Effekte möglich, und es zeigt sich ein größerer Einfluss durch die Modifizierung der Seitenkette als durch die Substitution des Chromanolringsystems.

Das Manuskript 4 zeigt das Potential der LCM zur Regulation des lipidtropfenassoziierten Proteins PLIN2 auf und untersucht die Interaktion der LCM mit koinkubierten Fettsäuren. Es wird deutlich, dass die LCM eine positive Wirkung auf die Überlebensfähigkeit der Zellen ausüben und dass dies in Abhängigkeit von der Regulation von PLIN2 erfolgt. Diese Ergebnisse deuten, ebenso wie die Daten von WALLERT *et al.* (2014a) und des Manuskriptes 1, auf eine mögliche protektive Wirkung der LCM im Kontext der zellulären Lipidhomöostase hin. Diese Daten unterstützen also T3.

Das zweite hier untersuchte Themengebiet ist das antiinflammatorische Potential der LCM. Hierbei ermöglicht das optimierte Protokoll aus Manuskript 2 die zuverlässige Messung der LPS-induzierten NO-Produktion. Vor allem das Potential antiinflammatorischer Testsubstanzen kann besonders gut erfasst werden. Somit wurde die Basis für eine optimale, effiziente und einfache NO-Messung mittels Griess-Assay gelegt. Dieses optimierte Protokoll wurde bereits in einem Kooperationsprojekt eingesetzt, welches zeitnah in eine Publikation münden wird (Hans-Knöll-Institut Jena, Toni Neuwirth und Christian Hertweck).

Anwendung findet diese Methode auch in den Manuskripten 1 und 3, die unter anderem die LPS-induzierte Inflammation untersuchen. Beide Manuskripte beschreiben einheitlich die antiinflammatorische Wirkung der LCM. Dabei spielt die Form der LCM (bereits untersucht α- und δ-LCM) eine untergeordnete Rolle. *In-vivo*-Studien am murinen Peritonitismodell, die von Andreas Koeberle (Institut für Pharmazie, Friedrich-Schiller-Universität Jena) durchgeführt wurden, stimmen ebenfalls sehr gut hiermit überein (unveröffentlichte Daten, persönliche Mitteilung). Auch er berichtet von ausgeprägten, antiinflammatorischen Eigenschaften der α-13'-COOH und der GA. Der Einfluss der LCM auf die Inflammation wird damit weiterhin bestätigt und eine sehr gute Übertragbarkeit der *In-vitro*- zu den *In-vivo*-Daten wird deutlich.

Auch hier deutet sich die Richtigkeit von T3 an.

Diese Erkenntnisse bereiten den Weg zur Aufklärung der zugrundeliegenden Signaltransduktion und damit zur Beantwortung von F2 und F3. Folgender Vergleich könnte hilfreich sein, um Ideen für die beteiligten Signalwege zu generieren. In der Literatur könnten Substanzen mit ähnlicher biologischer Wirkung beschrieben sein, wie sie für die LCM bislang bekannt ist. Falls für diese Substanzen die beteiligte Signaltransduktion bereits aufgeklärt ist, könnte diese auch für die LCM verantwortlich sein. Je mehr biologische Effekte der LCM also bekannt sind, umso präziser kann der Vergleich mit der Literatur durchgeführt werden. Derzeit bearbeiten zwei weitere Doktoranden der Arbeitsgruppe (Martin Schubert und Stefan Kluge) dieses Thema. Mit unterschiedlichen Methoden, wie Inhibitorversuchen, Transfektionsansätzen, Array-Experimenten und der Analyse der Proteinexpression von Signalproteinen liefern beide neue Erkenntnisse zu diesem Teilaspekt der LCM-Charakterisierung.

Die pharmazeutische Nutzung der LCM oder strukturähnlicher Verbindungen könnte eine Weiterentwicklung des Gesamtprojekts darstellen. Diese Arbeit legt dafür einen Grundstein, denn zunächst müssen die funktionell relevanten Substrukturen der LCM bekannt sein (siehe Manuskript 1). Für eine ausreichende Wirkungsabschätzung des potentiellen Medikaments ist das Wissen um die biologische Funktion der LCM essentiell. Diese wird in den Manuskripten 1, 3 und 4 untersucht. Die Wirkung des Pharmazeutikums könnte sowohl eine spezifische Verstärkung als auch eine Hemmung der biologischen LCM-Effekte sein. Umgesetzt wird diese Idee bereits von Andreas Koeberle für die Hemmung der 5-LO. Er und auch andere (JIANG *et al.* 2011) zeigten bereits, dass die GA und α-13'-COOH die Enzymaktivität effektiver hemmen als der synthetische Inhibitor Zileuton. Daher scheint dieser Ansatzpunkt vielversprechend.

7.2 Limitationen der Studien und mögliche Lösungsansätze

Zur realistischen Einschätzung der eigenen Daten ist ihre kritische Betrachtung sinnvoll. Dies erfolgt zunächst für die einzelnen Manuskripte, wobei Ideen für mögliche Lösungsansätze und Erweiterungen der Studiendesigns jeweils aufgezeigt werden. Im Anschluss werden allgemeinere Punkte diskutiert, die alle hier vorgestellten Studien betreffen.

7.2.1 Betrachtung der Originalarbeiten

7.2.1.1 Manuskript 1

Das Manuskript 1 zeigt, dass für die LCM-spezifische Regulation die gesamte chemische Struktur benötigt wird. Offen ist dabei noch die Frage, welchen Einfluss die Sättigung der Seitenkette hat. Strukturell ähnelt die GA dem im Manuskript 1 eingesetzten δ-13'-COOH, wobei sich die beiden Substanzen durch die Sättigung ihrer Seitenkette unterscheiden. Ein Vergleich mit den Daten aus Projekt 1, das die antiinflammatorische Wirkung der GA beschreibt, lässt die Schlussfolgerung zu, dass die Sättigung der Seitenkette eine untergeordnete Rolle spielt, da die GA-Effekte den LCM-Effekten in Richtung und Effektgröße ähneln. Zur Überprüfung können Experimente für die LCM und GA parallel durchgeführt werden, die dann einen direkten Vergleich der Daten zulassen.

Pasquale Richomme (Universität von Angers, Frankreich) gehört zum LCM-Netzwerk und hat eine umfangreiche Substanzdatenbank mit LCM-ähnlichen Verbindungen aufgebaut. Diese

Substanzen könnten die im Manuskript 1 gezeigte Strukturabhängigkeit bestätigen und weiter spezifizieren. Eventuell kann dies der Entwicklung von LCM-ähnlichen Pharmazeutika dienlich sein (siehe Kapitel 7.1.3).

Neben dem Einfluss von LCM-Substrukturen ist es denkbar, dass auch die räumliche Struktur der LCM auf ihre biologische Aktivität Einfluss nimmt. Wie in der Einleitung bereits erwähnt wurde, werden alle Experimente dieser Arbeit mit semisynthetischen Metaboliten durchgeführt. Diese besitzen eine hohe Reinheit und liegen als Enantiomergemisch vor. Die Frage, ob die Effektivität der enantiomerreinen LCM höher ist, muss derzeit offenbleiben, da ihre Synthese höchst anspruchsvoll und derzeit nicht durchführbar ist (persönliche Mitteilung von Marc Birringer). JANG *et al.* (2016) lieferten aber bereits erste Hinweise, dass die Gewinnung der LCM (ob semisynthetisch (Enantiomergemisch) oder durch Isolation aus Zellkulturmedium (enantiomerrein)) keinen Einfluss auf ihre Effektivität hat.

7.2.1.2 Manuskript 2

Untersuchungen zur Regulation der Inflammation durch die LCM machen deutlich, dass die RAW264.7-Makrophagen ein spezifisches und optimiertes Inkubationsschema benötigen, um die optimale Antwort auf den inflammatorischen Stimulus (LPS) zu erzielen. Diese Erkenntnisse werden in Manuskript 2 zusammengefasst, welches Strategien zur Ermittlung des optimalen Inkubationsschemas aufzeigt. Der Fokus dieser Studie liegt auf der verbesserten Detektion von antiinflammatorischen Effekten. Ob diese Strategien auch auf proinflammatorische Substanzen übertragbar sind, muss im Einzelfall geprüft werden. Weiterhin ist noch offen, ob dieses Inkubationsschema auch für andere Zelllinien, wie z.B. murine J774-Makrophagen, anwendbar ist, da die Optimierungen bislang nur mit den RAW264.7-Makrophagen durchgeführt wurden.

7.2.1.3 Manuskript 3

Manuskript 3 charakterisiert die antiinflammatorischen Effekte von α-13'-COOH auf Cox2 und iNos in RAW264.7-Makrophagen. Die Untersuchungen zum zugrundeliegenden Mechanismus liefern den Hinweis, dass eine NFκB-Translokation keine Rolle spielt. Auf der Basis der durchgeführten Untersuchungen kann allerdings keine Aussage zur Änderungen in der NFκB-Aktivität oder der DNA-Bindung getroffen werden. Diese könnten über Aktivitätsassays oder durch den Einsatz eines *electrophoretic mobility shift assay* (EMSA) nachgewiesen werden. Im Moment ist demzufolge die Frage nach dem verantwortlichen Regulationsmechanismus noch zu beantworten.

Die Daten des Manuskriptes 3 stimmen sehr gut mit den Ergebnissen von CIFFOLILLI *et al.* (2015) überein, welche den Einfluss von α-13'-OH auf sehr ähnliche Parameter untersuchten. Interessant wäre der Einfluss der α-LCM auf weitere proinflammatorische Enzyme, wie z.B. die 5-LO oder die mikrosomale PGE_2-Synthase-1 (mPGES-1), die das Konversionsprodukt der COX zu PGE_2 umsetzt (PGH_2 zu PGE_2), welches wiederum für die klassischen Kennzeichen der Inflammation, wie Schmerz und Fieber verantwortlich gemacht wird (KOEBERLE & WERZ 2015). Für δ-13'-COOH und GA ist die Hemmung der 5-LO bereits beschrieben (JANG *et al.* 2016; JIANG *et al.* 2011). ALSABIL *et al.* (2016) untersuchten den Einfluss aller GA-Formen (α- bis δ-GA) auf die mPGES-1-Aktivität und beschrieben eine Hemmung in einem zellfreien Assay (IC_{50}-Werte: 2,0 - 7,8 µM, γ-

GA > β-GA > δ-GA > α-GA). Basierend auf den Ergebnissen des Manuskriptes 1 kann spekuliert werden, dass auch α-13'-COOH ähnliche Effekte vermittelt. Die Untersuchungen zur Regulation der 5-LO durch α-13'-COOH werden derzeit von Andreas Koeberle vorgenommen und zeitnah publiziert. Aus diesem Grund geht das Manuskript 3 nicht darauf ein.

7.2.1.4 Manuskript 4

Im Fokus von Manuskript 4 steht die Lipotoxizität, die durch eine Inkubation von THP-1-Makrophagen mit freien Fettsäuren nachgeahmt wird. Pathophysiologisch entsteht die Lipotoxizität bspw. durch eine ungehemmte Aufnahme von oxLDL und eine anschließende Freisetzung der Fettsäuren; daraus resultieren intrazelluläre, toxische Konzentrationen an freien Fettsäuren, die zum Absterben der Zellen führen (BROOKHEART *et al.* 2009). Das Manuskript 4 deckt durch die Inkubation mit freien Fettsäuren den zweiten Teil des pathophysiologischen Prozesses ab. WALLERT *et al.* (2014a) untersuchten den ersten Teil des Prozesses und zeigten, dass bei einer Inkubation von THP-1-Makrophagen mit 50 mg/l oxLDL durch die Metabolite eine Reduktion der oxLDL-Aufnahme vermittelt wird. Es liegt nahe, dass diese beiden Prozesse ineinandergreifen und bei einer Inkubation der Zellen mit erhöhter oxLDL-Konzentration eine Lipotoxizität durch die LCM vermindert werden kann. Dies kann in separaten Bestätigungsexperimenten geprüft werden. Dazu bietet sich eine Inkubationsmatrix aus steigenden Konzentrationen an oxLDL und LCM an (ähnlich, wie sie im Manuskript 4 für Stearinsäure und LCM angewendet wird).

Interessant wäre außerdem die Untersuchung der intrazellulären Fettsäuregehalte. Basis dafür sind Hinweise aus genomweiten RNA-Arrays, die eine LCM-vermittelte Induktion der Stearoyl-Coenzym-A-Desaturase (SCD)-Expression aufzeigen. Dieses Enzym ist für die Dehydrierung von Stearinsäure zu Ölsäure zuständig (IWAI *et al.* 2016). Es wurde gezeigt, dass die ungesättigte Ölsäure ein geringeres Potential zur Lipotoxizität besitzt als die gesättigte Stearinsäure (RABKIN *et al.* 2009). Wenn die eingesetzten LCM eine vermehrte Umsetzung der Stearinsäure zu Ölsäure bewirken, könnte dies die LCM-abhängige Hemmung der stearinsäurevermittelten Lipotoxizität erklären. Durch Untersuchungen der intrazellulären Fettsäuregehalte oder durch eine Aktivitätsmessung der SCD kann diese Vermutung überprüft werden.

Ein Ansatzpunkt für weitere Experimente ist der dem Manuskript 4 zugrundeliegende Mechanismus. Durch Kombinationsexperimente mit spezifischen Inhibitoren möglicherweise beteiligter Signalproteine oder auch mit Hilfe eines entsprechenden *Knockdowns* dieser Signalproteine, kann ihr Einfluss auf die LCM-vermittelten Effekte untersucht werden. Hinweise zu geeigneten Signalproteinen werden von den beiden anderen Doktoranden der Arbeitsgruppe geliefert. Um diesen Arbeiten nicht vorzugreifen, bleiben die Zielproteine an dieser Stelle unbenannt.

7.2.2 Betrachtung des gewählten Modells

Die experimentellen Studien dieser Arbeit liefern wichtige Einblicke in die biologischen Effekte der LCM, wobei diese Ergebnisse zunächst nur unter den jeweils spezifischen Bedingungen gelten.

7.2.2.1 Einsatz der LCM im *In-vitro*-Modell

Alle Arbeiten werden *in vitro* durchgeführt. Die verhältnismäßig simple Zellkultur wird der Komplexität des Organismus jedoch nicht gerecht. Daher können die *In-vitro*-Ergebnisse nicht direkt auf systemische Prozesse und Regulationen übertragen werden.

Die Wahl der verwendeten Zellen basiert auf dem Wunsch, vorrangig humane Zellen zu verwenden, um die Übertragbarkeit auf den Menschen zu erleichtern. Außerdem sollen Zellen eingesetzt werden, die in der Atherogenese eine entscheidende Rolle spielen. Die Wahl fällt hierbei auf Makrophagen, die durch ihre ungehemmte Aufnahme von oxLDL zu Schaumzellen transformieren, durch Überladung mit Lipiden schließlich in den Zelltod gehen und den nekrotischen Lipidkern der atherosklerotischen Plaques bilden (WALLERT *et al.* 2014b).

Für die Manuskripte 1 und 4 werden humane THP-1-Monozyten gewählt, welche mittels spezifischer Reagenzien (β-Mercaptoethanol und Phorbol-12-Myristat-13-Acetat (PMA)) zu Makrophagen ausdifferenziert werden können (AUWERX *et al.* 1992). Diese Zellen werden häufig als Modell für primäre Makrophagen verwendet, da sie diesen nach der PMA-Stimulation sehr ähnlich sind (LUND *et al.* 2016). THP-1-Makrophagen wurden in allen Untersuchungen eingesetzt, die sich mit der zellulären Lipidhomöostase beschäftigen.

Ein zweiter wichtiger Prozess in der Atherogenese ist die Regulation der Inflammation, welche in den Manuskripten 1 bis 3 untersucht wird. Humane Makrophagen stellen hierfür ein schlechtes Modell dar, da ihnen das funktionelle Enzym iNOS fehlt (GROSS *et al.* 2014). Somit können Untersuchungen zur Regulation der iNOS oder des inflammatorischen Mediators NO nicht in THP-1-Makrophagen durchgeführt werden. Aus diesem Grund wurden murine RAW264.7-Makrophagen als Modellsystem eingesetzt. Die Verwendung der Zellen für diesen Einsatzzweck ist bereits vielfach beschrieben (TAYLOR *et al.* 2003) und diese Zellen stellen ein etabliertes Modell dar.

7.2.2.2 Eingesetzte Konzentrationen der LCM

Die eingesetzte Konzentration der LCM (13'-OH: 10 µM, 13'-COOH: 5 µM) sollte kritisch betrachtet werden. Zur Bewertung, ob diese Konzentrationen physiologisch sind, muss derzeit der Vergleich mit den humanen Serumkonzentrationen herangezogen werden, da die Konzentrationen in den Zielgeweben und damit in den Zielzellen unbekannt ist (siehe Lösungsansätze unter Kapitel 7.3.4). GIUSEPPONI *et al.* (2017) zeigten kürzlich, dass α-13'-COOH im Serum von gesunden Freiwilligen basal in einer Konzentration von etwa 1,2 nM vorliegt. Nach einer Supplementation mit 1000 IU *RRR*-α-TOH für eine Woche konnten ~ 2,4 nM α-13'-COOH detektiert werden. Alle veröffentlichten Daten zu den Serumkonzentrationen der LCM beruhen auf der Messung von drei Probanden (normolipämisch, im Alter von 27 bis 37 Jahren, zwei männlich und eine weiblich; CIFFOLILLI *et al.* 2015; GIUSEPPONI *et al.* 2017; WALLERT *et al.* 2015). Diese Ergebnisse müssen daher durch weitere Messungen abgesichert werden (siehe Kapitel 7.3.3). Dennoch sind die Daten ausreichend valide, um zu dem Schluss zu gelangen, dass die in den Manuskripten 1 bis 4 eingesetzten Konzentrationen supraphysiologisch sind (Vergleich der Größenordnung: nM *in vivo* und µM *in vitro*).

Die Serumkonzentrationen der LCM liefern zwar einen ersten Anhaltspunkt, inwiefern die *in vitro* eingesetzten Konzentrationen physiologisch sind, aber relevanter wären für einen Vergleich die

Konzentrationen in den Zielzellen *in vivo*, in diesem Falle in den Makrophagen der Arterien. Erste Versuche nähern sich diesem Ansatzpunkt, im dem die intrazellulär erreichbare Konzentration der LCM in unterschiedlichen Zellen ermittelt wird. Die Ergebnisse zu den RAW264.7- und den THP-1-Makrophagen wurden bereits unter Kapitel 7.1.2 beschrieben. Andreas Koeberle nähert sich der *In-vivo*-Situation etwas mehr an, in dem er *in vitro* primäre, polymorphkernige neutrophile Leukozyten (PMNL) mit 100 nM α-13'-COOH inkubiert. Er gibt eine intrazelluläre Konzentration von ~ 50 µM α-13'-COOH an (unveröffentlichte Daten, mündliche Mitteilung von Andreas Koeberle).

Offenbar bestehen relativ große Unterschiede in der Aufnahmekapazität der Zellen (100 nM extrazellulär führt zu 50 µM intrazellulär in PMNL, bzw. 5 µM extrazellulär führt zu 4 µM intrazellulär in THP-1-Makrophagen). Hierbei können mehrere Faktoren eine Rolle spielen. Zum einen werden für die Untersuchungen Zellen unterschiedlicher Herkunft (primäre Zellen *vs.* Zelllinie) und unterschiedlicher Art (PMNL *vs.* Makrophagen) verwendet, die unter Umständen unterschiedliche Affinitäten zu den LCM aufweisen. Dies wird auch in den Aufnahmeexperimenten aus Projekt 2 deutlich, bei denen solche Unterschiede zwischen THP-1-Makrophagen und RAW264.7-Makrophagen gezeigt werden (siehe Kapitel 7.1). Weiterhin könnten die unterschiedlichen Inkubationskonzentrationen einen Einfluss auf die Aufnahmeeffizienz haben. Abschätzen kann man dies durch konzentrationsabhängige Aufnahmeexperimente, wie sie im weiteren Verlauf der Diskussion erläutert werden (siehe Kaptiel 7.3.8).

Die Analytik der gerade beschriebenen Ergebnisse erfolgte in unterschiedlichen Laboren, mit unterschiedlichen Proben und wurde mit Hilfe unterschiedlicher Verfahren durchgeführt. Um sicherzustellen, dass die Analyse der Proben gut übereinstimmt, könnte dieselbe Probe laborübergreifend vermessen und die Ergebnisse im Anschluss verglichen werden. Dies ist für andere Laborwerte in Form von Ringversuchen Standard (ALBANO & CATEN 2014).

Zur endgültigen Plausibilitätsprüfung der Daten wären allerdings Informationen zu den tatsächlichen Gewebekonzentrationen der LCM hilfreich. Unter Kapitel 7.3.4 werden Strategien dazu beschrieben.

Der Einsatz von supraphysiologischen Konzentrationen ist für *In-vitro*-Experimente allerdings nichts Ungewöhnliches. Häufig werden Substanzen deshalb *in vitro* in höheren Konzentrationen eingesetzt, um grundsätzliche Wirkprinzipien aufzuklären. In dem Falle dieser Arbeit sollte zudem die Vergleichbarkeit zu vorangegangenen Studien gewährleistet werden. Künftig sollen die Untersuchungen teilweise unter Verwendung von niedrigeren LCM-Konzentrationen durchgeführt werden (z.B. 100 nM, wie von Andreas Koeberle bereits vorgeschlagen).

7.2.2.3 Art und Form der LCM

Die eingesetzten LCM liegen als Enantiomergemisch vor und entsprechen damit nicht den physiologischen Metaboliten. Falls enantiomerreine LCM mit vertretbarem Aufwand synthetisiert werden könnten, wäre die Überprüfung möglich, ob die in diesen Studien eingesetzten, verhältnismäßig hohen Konzentrationen zur Erzielung der Effekte nötig sind, da die „Effektorstruktur" nur ein Achtel des vorliegenden Substanzgemisches ausmacht. Wie bereits erwähnt wurde, legen die Daten von JANG *et al.* (2016) einen anderen Schluss nahe (siehe Kapitel 7.1).

Der Metabolismus von Vitamin E verläuft in Abhängigkeit von der Vitaminform in unterschiedlicher Effektivität (Manuskript 6). Die in dieser Arbeit dargestellten Ergebnisse wurden mit α- und δ-LCM erzielt. Physiologisch ist allerdings α-TOH besonders gut vor dem Abbau geschützt (LEONARD *et al.* 2005) und δ-TOH spielt in der Ernährung eine untergeordnete Rolle (MAHABIR *et al.* 2008). Es wäre daher zu erwarten, dass die Serumkonzentrationen der α- und δ-LCM gering ist. Die Untersuchung der γ-LCM könnte physiologisch bedeutsamer sein, da γ-TOH insbesondere in den USA besonders reichlich verzehrt wird (JIANG *et al.* 2001) und aufgrund der höheren Abbaurate für γ-TOH, eine höhere Konzentration an γ-LCM denkbar wäre. Eine Bestätigung in Form einer γ-LCM-Serumanalytik steht noch aus. Die Untersuchung der γ-LCM *in vitro* gestaltet sich derzeit schwierig, da die Synthese der γ-LCM bislang nicht im größeren Maßstab möglich ist. Kürzlich wurde aber die γ-GA aus *Garcinia amplexicaulis* (mit einer bescheidenen Ausbeute von 6 mg γ-GA aus 270 g Rindenmaterial) isoliert (ALSABIL *et al.* 2016). Parallel zur Semisynthese der α- und δ-LCM könnten, ausgehend von der γ-GA, die γ-LCM synthetisiert werden. Dies ist sicherlich ein interessanter Ansatz, welcher das Wissen um die LCM erweitern würde.

Die detaillierte Untersuchung der α-LCM ist dennoch lohnenswert: es kann spekuliert werden, dass die Favorisierung von α-TOH in der Leber, bzw. die feinmaschige Regulation des α-TOH-Abbaus auf der Tatsache basiert, dass α-13'-COOH der aktive, hormonähnliche Metabolit des α-TOH ist. Ob diese Vermutung sich bewahrheitet, wird sich in den nächsten Jahren bzw. Jahrzehnten zeigen, wenn das Wissen um die LCM vertieft wurde.

7.3 Physiologie der LCM

Beinahe alle hier diskutierten Limitationen der Studien zielen auf die Unterschiede zwischen den Versuchsbedingungen und der physiologischen Situation ab. Trotzdem sind die bisherigen Ergebnisse von entscheidender Bedeutung, da z.B. die antiinflammatorische Wirkung der LCM *in vitro* und *in vivo* sehr gut übereinstimmen.

Für eine Anpassung der *In-vitro*-Experimente ist eine tiefgründige Aufklärung der LCM-Physiologie nötig. Im Moment sind hierfür noch zu viele Fragen offen: z.B. die Transportform der LCM im Blut, das Ort-Zeit-Profil bei der Entstehung der LCM im Körper, die Interaktion der unterschiedlichen Vitamin-E-Formen bei der Bildung ihrer Metabolite oder auch die Beeinflussung der LCM-Konzentration durch physiologische und pathophysiologische Zustände.

7.3.1 Analytik der LCM

Basis für beinahe alle weiterführenden Experimente stellt eine optimierte und zuverlässige Analytik der Metabolite dar. Marc Birringer und Alexander Maxones stellen hierfür eine Analytikplattform zur Verfügung. Sie nutzen erstmalig ein Hybrid-Festphasenextraktionssystem (*hybrid solid phase extraction*, Hybrid-SPE), welches die Phospholipidfraktion der Probe bindet und somit einen relevanten Störfaktor für die LC-MS-Analyse entfernt (Projekt 2). Damit bleiben alle Metabolite und Vorstufen in der Probenmatrix enthalten und das Problem der Polaritätsunterschiede bei der Flüssig-Flüssig-Extraktion wird elegant umgangen. Weiterhin sollen alle Metabolite (SCM, LCM und TOH) in einem Lauf detektierbar sein. Problematisch sind dabei,

wie schon in der Einleitung erwähnt wurde (siehe Kapitel 2.1), die großen Konzentrationsunterschiede insbesondere zwischen den Metaboliten und ihren Vorstufen, wenn z.B. Serum vermessen werden soll. Eine Kombination dreier massenspektrometrischer Einheiten könnte eine zusätzliche Fraktionierung der Proben die Messung der TOH-Konzentration ermöglichen. Geplant ist hierbei die Vermessung eines TOH-Fragments, dessen Konzentration innerhalb des Detektionsbereichs liegt und das einen linearen Zusammenhang zur TOH-Konzentration zeigt. Falls dies erfolgreich wäre, könnten in einem relativ kleinen Probenvolumen (100 µl für Blutproben) alle Metabolite und Vorläufer in einem chromatographischen Lauf detektiert werden. Für den routinemäßigen Einsatz in großen Humanstudien muss das benötigte Probenvolumen allerdings weiter verringert werden, da möglichst viele Messungen mit dem meist limitierten Probenmaterial durchgeführt werden sollen.

7.3.2 Wahl der Testsysteme

Der Einsatz der LCM im Tiermodell würde einige Limitationen der *In-vitro*-Experimente überwinden. Wichtig sind hierbei die richtige Dosisfindung (siehe Kapitel 7.3.3 bis 7.3.5), die Art der Applikation (siehe Kapitel 7.3.7), der Zeitpunkt des Versuchsendes (siehe Kapitel 7.3.5) sowie die Wahl eines geeigneten Modellsystems. Dabei müssen die Fragen nach dem Modelltier, dem genetischen Hintergrund und der untersuchten Erkrankung geklärt werden. Die Basis dafür ist eine gute Charakterisierung der LCM, um die genannten Entscheidungen sinnvoll treffen zu können. Dafür wiederum können die *In-vitro*-Experimente hilfreich sein, um erste Hinweise z.B. auf ein geeignetes Modellsystem zu gewinnen. So erscheinen Modelle, bei denen die Regulation der Inflammation eine Rolle spielt auf Grundlage der hier vorgestellten Daten vielversprechend (zur Datenlage siehe Kapitel 7.1, für künftige Projekte siehe Kapitel 7.4.2).

Die direkte Übertragbarkeit der Daten aus dem Maus- oder Rattenmodell auf den Menschen ist aber dennoch nicht gegeben. Eine Übersicht zu den Ähnlichkeiten und den Unterschieden des Vitamin-E-Metabolismus bei Nagern und Menschen kann der Dissertation von WALLERT (2014) entnommen werden. Die Homologie in der Beteiligung der Enzymsysteme und der entstehenden Metabolite wird dort deutlich. Im Menschen wurde bislang hingegen eine größere Anzahl an Konjugaten beschrieben. Ob diese Konjugate (z.B. Sulfate) im Nager nicht entstehen oder bisher lediglich nicht nachgewiesen wurden, bleibt noch zu klären.

Mit dem derzeitigen Wissen sind Untersuchungen, welche auf einer Applikation der LCM im Menschen beruhen, nicht verantwortbar, da die Sicherheit für die Probanden derzeit nicht gegeben ist. Zu komplex sind die möglichen Interaktionen der LCM im Organismus und zu gering ist im Vergleich dazu das derzeitige Wissen um die biologischen Effekte der LCM. Aus diesem Grund beschränken sich die Möglichkeiten der Humanstudien zurzeit auf der Charakterisierung der Transportwege der natürlich gebildeten LCM, der physiologischen LCM-Serumkonzentration im gesunden, wie im pathologischen Zustand und der Regulation des Vitamin-E-Metabolismus im basalen und Vitamin-E-supplementierten Zustand.

7.3.3 Physiologische und pathophysiologische Serumkonzentrationen

Das Screening der LCM-Serumspiegel von Gesunden und Erkrankten würde einen guten Einblick über die physiologischen Konzentrationen der LCM geben und so möglicherweise eine

Anpassung der eingesetzten Konzentration bei zukünftigen *In-vitro*- und *In-vivo*-Experimenten ermöglichen. Die bislang publizierten LCM-Serumkonzentrationen basieren auf der Vermessung von drei Probanden (CIFFOLILLI *et al.* 2015; GIUSEPPONI *et al.* 2017; WALLERT *et al.* 2014a). Zur Absicherung dieser Daten wäre daher eine größere Probenzahl essentiell. Weiterhin würden die Informationen aus Erkrankten unter Umständen Hinweise liefern, welche Mechanismen an der Bildung, dem Transport oder der Verteilung der LCM beteiligt sind. In einer kleinen Vorabstudie wurden Serumproben von Gesunden (Projekt 2) und von Hypercholesterolämiepatienten vermessen. Die Ergebnisse sind allerdings noch ausstehend. GIUSEPPONI *et al.* (2017) untersuchten Patienten mit chronischem Nierenversagen (*chronic kidney disease*, CKD) und fand für α-13'-COOH und α-13'-OH (jeweils einschließlich nicht identifizierter Peaks, die Isomere darstellen könnten) verringerte Werte. Dies deutet darauf hin, dass in CKD-Patienten die ersten Schritte des enzymatischen Abbaus gestört sein könnten.

7.3.4 Gewebeverteilung

Für die Optimierung der *In-vitro*-Versuche ist das Wissen um die LCM-Konzentration in den Zielgeweben oder den Zielzellen noch wesentlicher. Diese kann in Versuchstieren untersucht werden. Hierfür können die Konzentrationen basal, nach Vitamin-E-Supplementation oder nach LCM-Verabreichung gemessen werden, um z.B. einen Eindruck über die Affinität der Metabolite zu möglichen Speichergeweben zu gewinnen. Für den Bezug zu den hier vorgestellten Daten ist die Untersuchung von z.B. Aorten oder spezifischer, der enthaltenen Makrophagen, relevant. Weitere interessante Gewebe wären Muskel und Fettgewebe als Hauptspeicherort für Vitamin E (BJØRNEBOE *et al.* 1990) sowie die Leber, welche als Kurzzeitspeicher für Vitamin E dient (MACHLIN & GABRIEL 1982) und überwiegend für dessen Metabolisierung verantwortlich ist (BARDOWELL *et al.* 2012). Weiterhin ist das Gehirn interessant, da bei Mangel an Vitamin E kognitive Dysfunktionen beobachtet werden (FUKUI *et al.* 2015). Unter Supplementation steigt die α-TOH-Konzentration im Herzen dosisabhängig (MUSTACICH *et al.* 2007), weshalb Veränderungen in der LCM-Konzentration vermutet werden könnten. Aktuelle Analysen von GIUSEPPONI *et al.* (2017) zeigen, dass CKD-Patienten einen beeinträchtigten Vitamin-E-Metabolismus besitzen. Daher erscheint die Untersuchung der LCM-Konzentration in der Niere aufschlussreich. Aktuelle, unveröffentlichte Daten von Maria Wallert deuten auf eine Zunahme des Milzgewichtes unter α-13'-COOH-Applikation hin (unveröffentlichte Daten, mündliche Mitteilung). Daher scheint dies ebenfalls ein lohnenswertes Zielgewebe darzustellen.

Um die Relevanz im Menschen einzuschätzen, wären die humanen Gewebekonzentrationen der LCM höchst interessant. Aufgrund offensichtlicher ethischer Hindernisse für solche Messungen bei gesunden Probanden sollte geprüft und diskutiert werden, ob eine LCM-Analyse von Obduktionsmaterial ausreichende Informationen liefert. Alternativ kann in Kooperation mit einem Klinikum Operationsmaterial gewonnen werden. Dies bietet den Vorteil, dass unter Umständen das Ernährungsverhalten der Patienten abgefragt werden kann und damit mehr Informationen vorliegen als beim Obduktionsmaterial. Für erste Hinweise zu den humanen Gewebekonzentrationen können beide Probenarten sehr gut genutzt werden. Allerdings sollte beachtet werden, dass diese unter Umständen von den Konzentrationen in gesunden Probanden abweichen können.

Neben der Gesamtgewebekonzentration kann auch die zellspezifische und die subzelluläre Verteilung der LCM aufgeklärt werden, welche in F1 adressiert wurde. Durch die Isolation von Zelltypen aus Geweben könnten spezifische Zielzellen identifiziert und in weiteren *In-vitro*-Studien adressiert werden. Bei der Aufklärung der subzellulären Verteilung könnten Speicher- und Wirkorganellen identifiziert werden. Der Vergleich von extrahepatischen Zellen zu Leberzellen lässt unter Umständen spezifische Verteilungsmuster erkennen und könnte erste Einblicke in biologische Mechanismen erlauben. So gibt das LCM-Verteilungsmuster in Leberzellen z.B. Hinweise auf den Ort des Vitamin-E-Metabolismus (MUSTACICH *et al.* 2010). Sollte sich in extrahepatischen Zellen das Verteilungsmuster unterscheiden, könnte dies möglicherweise den Ort der LCM-Aktivität anzeigen und Untersuchungen dort lokalisierter Stoffwechselvorgänge ermöglichen. Für andere Metabolite ist Ähnliches bereits beschrieben: so akkumulieren von epigenetischen Enzymen benötigte Metabolite (*epigenetic enzyme required metabolites*, EERM) am Ort ihrer zukünftigen Verwendung (ZHAO *et al.* 2016). Die Überprüfung dieser These ist sicherlich lohnenswert.

Zur praktischen Umsetzung liegen zwei Konzepte vor, nämlich die Fraktionierung und die RAMAN-spektroskopische Analyse der Zellen. Die Fraktionierung der Zellen stellt dabei den klassischen Ansatz dar. MUSTACICH *et al.* (2010) fraktionierte die untersuchten Zellen in Mikrosomen, Peroxisomen und Mitochondrien. Basierend auf unveröffentlichten Arbeiten von Martin Schubert wäre die Untersuchung der nukleären Fraktion und aufgrund der lipophilen Eigenschaften der LCM die Analyse der Membranfraktion interessant. Die Anwendung verschiedener Verfahren, wie die Dichtegradienten- oder differentielle Zentrifugation sowie der Einsatz von speziellen Homogenisatoren (DOUNCE-Homogenisator zum spezifischen Erhalt der intakten Organellen, z.B. Nuclei; GRAHAM 2002) ermöglichen die subzelluläre Fraktionierung. Die Überprüfung der Reinheit der einzelnen Fraktionen kann mittels Western-Blot oder auch durch eine massenspektrometrische Analyse anhand von spezifischen Markerproteinen erfolgen (DRISSI *et al.* 2013).

Darüber hinaus könnte eine RAMAN-spektroskopische Analyse von Zellen, die zuvor mit deuterierten LCM inkubiert wurden, einen Einblick in das intrazelluläre Verteilungsmuster bieten. In einem Kooperationsprojekt mit Clara Stiebing (Leibniz-Institut für Photonische Technologien, Jena) wurde ein ähnlicher Ansatz für deuterierte Fettsäuren, welche in oxLDL eingebracht und dann THP-1-Makrophagen angeboten wurden, bereits erfolgreich getestet (STIEBING *et al.* 2017).

7.3.5 Pharmakokinetik der LCM

Zur Festsetzung sinnvoller Behandlungs- oder Inkubationszeiten ist es wichtig, die LCM-Bildung und -Verteilung näher zu kennen. Zur Aufklärung der LCM-Pharmakokinetik wird den Testpersonen oder -tieren eine definierte Dosis an Vitamin E verabreicht, zu unterschiedlichen Zeitpunkten werden Blut oder im Falle der *In-vivo*-Studien Gewebe entnommen und anschließend die darin enthaltene Konzentration der LCM bestimmt. Aus diesen Daten kann ein Zeit-Ort-Verteilungsprofil erstellt werden. Diese Informationen sind für nachfolgende *In-vivo*-Studien von herausragender Bedeutung. Nur so kann sichergestellt werden, dass der Endpunkt der Studie zeitlich optimal gewählt wird und die LCM am gewünschten Wirkort zur Verfügung stehen.

Denkbar wäre außerdem der Einsatz von deuteriertem Vitamin E, um ausschließlich die neugebildeten Metabolite zu erfassen. Diese Experimente würden die Daten von TRABER *et al.* (2017)

um das Wissen für die LCM erweitern. Sie verabreichten Testpersonen 15 mg deuteriertes *RRR*-α-TOH und analysierten innerhalb der nachfolgenden 72 h mehrfach die Serum- und Urinkonzentrationen der SCM (α-CEHC und α-CMBHC).

In sogenannten Dosis-Wirkungsstudien kann die Konzentration der gebildeten LCM (im Blut und Geweben) in Abhängigkeit von der zur Verfügung stehenden Vitamin-E-Konzentration ermittelt werden. Die Erkenntnisse dieser Studien dienen der Dosisfindung in kommenden *In-vivo*-Studien.

Darüber hinaus ist die Durchführung solcher Studien auch *in vitro* essentiell, da auf diese Weise eingeschätzt werden kann, ob in Abhängigkeit von der eingesetzten Zelllinie ein Metabolismus der Testsubstanzen stattfindet. Für Leberzellen ist dieser bereits beschrieben (BIRRINGER *et al.* 2010). Aktuelle Daten von Marc Birringer deuten darauf hin, dass α-TOH von THP-1-Makrophagen, RAW264.7-Makrophagen und humanen Fibroblasten nicht abgebaut wird, wobei hingegen α-13'-OH zu α-13'-COOH abgebaut wird (siehe Kapitel 7.1.2). Ob eine weitere Metabolisierung von α-13'-COOH stattfindet, kann aufgrund fehlender Daten derzeit nicht eingeschätzt werden. Allerdings deuten die aktuellen *In-vitro*-Daten eher auf eine intrazelluläre Akkumulation von α-13'-COOH hin (Projekt 2; siehe außerdem Kapitel 7.3.8). Falls dies der Fall wäre und eine Bioaktivierung von α-13'-OH zu α-13'-COOH zur vollen biologischen Effektivität nötig wäre, wäre die Kenntnis der verantwortlichen Enzyme wertvoll. Möglicherweise gibt die subzelluläre Verteilung der LCM (siehe Kapitel 7.3.4) erste Hinweise auf die beteiligten Enzyme.

7.3.6 Charakterisierung der Regulation des Metabolismus

Die Identifizierung der beteiligten Enzyme kann über Inhibitoren oder auch spezifische Transfektionsansätze erfolgen. Um die Zahl der möglichen Enzyme einzugrenzen, kann das intrazelluläre Verteilungsprofil der Metabolite (siehe Kapitel 7.3.4) hilfreich sein. So lässt der Vergleich der Hauptbildungsorte mit den potentiellen Enzymkandidaten eventuell eine Schnittmenge entstehen, die dann experimentell untersucht werden kann.

Vielversprechende Kandidaten sind hierbei Enzyme, für die Substrate mit einer Strukturähnlichkeit zu den LCM beschrieben sind. Die Beteiligung von CYPs am initialen Schritt des Vitamin-E-Metabolismus ist bereits vielfach dokumentiert (siehe Kapitel 1.9). Aus chemischer Sicht sind an der Bildung von α-13'-COOH aus α-13'-OH zunächst eine Aldehyd- und dann eine Alkoholdehydrogenase beteiligt (MUSTACICH *et al.* 2010). Im Anschluss könnte aufgrund der hohen Strukturähnlichkeit der Abbau der Seitenkette unter Beteiligung der Enzyme für den Abbau der verzweigtkettigen Fettsäuren ablaufen (MUSTACICH *et al.* 2010). Dies liefert erste Anhaltspunkte zur Identifizierung der spezifischen Enzyme.

Auf Basis des unter Kapitel 7.1.2 beschriebenen Metabolismus von α-13'-OH zu α-13'-COOH in den *In-vitro*-Modellen muss derzeit davon ausgegangen werden, dass die beschriebenen Effekte für α-13'-OH durch eine Interaktion oder Überlagerung der Effekte von α-13'-OH selbst und dem neugebildeten α-13'-COOH entstehen. Falls die gerade erläuterten Anstrengungen zur Identifikation der verantwortlichen Enzyme erfolgreich wären, könnte der Einsatz von spezifischen Inhibitoren oder einen siRNA-vermittelten *Knockdown* Klarheit über die Einflussgröße der jeweiligen Metabolite bringen.

Durch den Vorschlag von Marc Birringer die LCM chemisch zu modifizieren, um ihren

intrazellulären Abbau zu unterbinden, ergibt sich ein weiterer Lösungsansatz zu dieser Problematik. Dies könnte durch eine Substitution einzelner Wasserstoffatome durch Fluoratome erfolgen, wodurch die LCM der β-Oxidation nicht mehr zugänglich wären. Diese LCM wären dann auch für den Einsatz in Vitamin-E-metabolisierenden Zellen geeignet. Über diesen Ansatz könnte aufgeklärt werden, ob die α-13'-OH-Effekte auf der Umwandlung zu α-13'-COOH beruhen, oder ob die Hydroxychromanole selbst auch eine Bioaktivität besitzen.

Parallel zum Abbau des Vitamin E verläuft die Konjugation der γ- und δ-Metabolite mit z.B. Sulfatgruppen (Nachweis *in vitro* in A549-Zellen, siehe Kapitel 1.9; JIANG *et al.* 2007). JIANG *et al.* (2008) zeigten, dass die sulfatierten Metabolite keine biologische Aktivität aufweisen (siehe Kapitel 2.4.1). Die Konjugation der α-LCM ist bislang noch nicht beschrieben. Unveröffentlichte Daten weisen aber darauf hin, dass die α-LCM im Serum sowohl als Sulfate als auch als Glucuronide vorliegen (wobei keine weiteren Dekonjugationsenzyme getestet wurden; Projekt 2). Ungeklärt ist außerdem, ob die α-LCM *in vitro* in den hier eingesetzten Zelllinien (THP-1- und RAW264.7-Makrophagen) konjugiert werden und ob zellspezifische Unterschiede vorliegen. Sollte sich herausstellen, dass *in vitro* keine α-LCM-Konjugate gebildet werden, liegen die Metabolite in biologisch aktiver Form vor. Falls *in vivo* ein größerer Anteil der Metabolite als Konjugate und damit in biologisch inaktiver Form vorliegt, würde dies die Übertragbarkeit der Daten von *in vitro* auf *in vivo* erschweren.

7.3.7 Transport der LCM im Organismus

Das Wissen um die physiologische Transportform der LCM im Blut und ihre Verteilung im Organismus ist für eine Anpassung der Inkubationsbedingungen *in vitro* essentiell. Ausgehend von der Transportform für Vitamin E (Manuskript 6) und der lipophilen Eigenschaften der LCM kann spekuliert werden, dass die LCM im Blut ebenfalls über die Lipoproteine transportiert werden.

Für die Aufklärung kann humanes Plasma per sequentieller Ultrazentrifugation in die Lipoproteinfraktionen (Chylomikronen, VLDL, LDL, HDL und lipoproteindepletiertes Plasma) getrennt werden und diese anschließend auf ihren LCM-Gehalt hin untersucht werden. Damit kann möglicherweise eine Aussage über die vorrangige Transportform für die LCM im Blut getroffen werden. Diese Versuche können unsupplementiert oder nach Gabe von Vitamin-E-Präparaten erfolgen, um zeit- und konzentrationsabhängige Unterschiede zu erfassen.

Eventuell existieren auch spezifische LCM-Bindeproteine, die deren Transport vermitteln. In extravaskulären Flüssigkeiten mit geringem Lipoproteingehalt vermittelt z.B. Afamin den Transport von α- und γ-TOH (HUBALEK *et al.* 2014). Daher wäre dieser Fall auch für die LCM denkbar. Ähnlich zu den *Fishing*-Experimenten könnte die Bindung von vaskulären oder im weiteren Verlauf auch von extravaskulären Proteinen ermittelt werden. Denkbar wäre außerdem eine unspezifische Bindung der LCM an Serumproteine.

Ziel der Untersuchungen der vorliegenden Arbeit ist das Nachahmen der physiologischen Bedingungen in den *In-vitro*-Versuchen. Es besteht die Möglichkeit, dass die LCM in Abhängigkeit von ihrer Applikationsform unterschiedliche Effekte vermitteln. Angenommen, die LCM würden vorrangig über LDL transportiert, dann würden sie auch über den LDL-typischen Internalisierungsweg in die Zelle aufgenommen und dann möglicherweise aus den Lysosomen freigesetzt

werden. Voraussichtlich unterscheidet sich dieser Prozess von den Prozessen, wie sie durch die bisherigen Applikationen (serumfreie Inkubation im Lösungsmittel Dimethylsulfoxid (DMSO)) herbeigeführt wurden.

7.3.8 Zelluläre Aufnahme

Die Frage nach der zellulären Aufnahme der LCM (F1) bleibt Gegenstand der aktuellen Forschung und ist vermutlich, wie in 7.3.7 beschrieben, von der Applikationsform abhängig. Gerade für die Interpretation der *In-vitro*-Daten ist dieses Wissen allerdings essentiell. Aus vorläufigen Daten kann geschlussfolgert werden, dass ein aktiver Aufnahmeprozess zur Anreicherung der LCM in den Zellen führt (DMSO-Applikation, serumfreie Inkubation), da für α-13'-COOH eine Aufnahmeeffizienz von etwa 78 % innerhalb von 24 h ermittelt wurde (Projekt 2). Damit liegt eine Aufnahme entgegen eines Konzentrationsgradienten vor und dies impliziert die Beteiligung eines aktiven Transporters. Weitere, unveröffentlichte Daten von Andreas Koeberle zeigen, dass GA am isolierten Enzym eine höhere Aktivität aufweist als α-13'-COOH, während im zellulären Enzym α-13'-COOH effektiver in der Regulation des gleichen Enzyms ist als GA. Daher scheinen Aufnahmeprozesse für α-13'-COOH die Regulationseffektivität zu beeinflussen.

Im Rahmen dieser Arbeit wurde ein Konzept erarbeitet, das die Aufnahmeprozesse der LCM in die Zellen näher charakterisiert. Die Aufnahmekinetik wird dabei zeit-, konzentrations- und temperaturabhängig untersucht, um eine endgültige Bestätigung über die Art des Aufnahmeprozesses (aktiv oder passiv) zu erhalten. Durch den Einsatz von spezifischen Inhibitoren könnte die Art des hypothetischen Transporters charakterisiert werden. So kann die ATP-Bereitstellung gehemmt werden (ROMERO-CANELÓN *et al.* 2012) oder die Beteiligung von Kotransportionen bestimmt werden. Dies erfolgt z.B. über Na^+-depletierte Medien (BHARDWAJ *et al.* 2005). Auch Inhibitoren für transporterunabhängige Aufnahmeprozesse können eingesetzt werden. So wären die energieabhängige Endozytose, die dynaminabhängige Endozytose oder auch die Makropinozytose lohnenswerte Ziele (MANN *et al.* 2016). Weitere relevante Informationen, z.B. zur Stabilität der LCM während der Inkubation, könnten durch eine Inkubation der LCM in Medium aber ohne Zellen gewonnen werden. Hierbei müsste allerdings beachtet werden, dass Interaktionen mit der Zellkulturgefäßoberfläche in diese Betrachtung miteinfließen. Diese Interaktion ist allerdings auch für Experimente mit Zellen zu erwarten, da auch in diesem Falle einige Bereiche der Zellkulturgefäßoberfläche zellfrei bleiben, z.B. insbesondere der Rand der Gefäße. Über eine Behandlung der inkubierten Zellen mit Trypsin könnte der Anteil der intrazellulären LCM ermittelt werden, da es denkbar ist, dass die LCM unspezifisch an die zelluläre Oberfläche gebunden sind. Bei diesem Ansatz muss allerdings beachtet werden, dass die Kurzzeitinkubation der Zellen mit Trypsin-PBS ein Ausströmen der aufgenommenen Substanzen verursachen kann, sodass der Einfluss der unspezifischen, zellulären Bindung überschätzt werden würde (TEUSCHER *et al.* 2017). Der zweite Teil der F1 zur subzellulären Verteilung der LCM wurde bereits unter Kapitel 7.3.4 ausführlich diskutiert.

7.4 Laufende Projekte zur Aufklärung der biologischen Effekte der LCM

Um einen umfassenden Überblick über das LCM-Projekt zu bieten, werden in den folgenden Abschnitten Forschungsvorhaben vorgestellt, die unter der Federführung der Autorin oder unter

ihrer Mitarbeit derzeit geplant oder durchgeführt werden.

7.4.1 Projekte auf zellbiologischer Ebene

Zur Erweiterung der hier vorgestellten Daten zur zellulären Lipidhomöostase wird auch der Export von Lipiden (in diesem Fall: Cholesterol) untersucht. Dabei ist das Projekt auf die Regulation des Cholesterolexporters ABCA1 durch die LCM fokussiert. Die Expression von ABCA1 wird durch die LCM auf mRNA- und Proteinebene gesenkt und erste Hinweise auf die zugrundeliegende Signaltransduktion wurden durch Stefan Kluge bereits gewonnen. Der Nachweis der funktionellen Relevanz, z.B. über den Cholesterolefflux steht noch aus. Im Rahmen eines Forschungsaufenthalts der Autorin in Innsbruck unter der Betreuung von Andreas Ritsch (Arbeitsgruppe Reverser Cholesterin Transport, Universitätsklinikum Innsbruck, Österreich) wurde der Efflux mit radioaktivmarkiertem Cholesterol für die primären, humanen Fibroblasten angepasst und durchgeführt. Dabei zeigten die LCM keinen Einfluss auf den Efflux, wobei auf Proteinebene unter gleichen Bedingungen kein ABCA1-Protein nachweisbar war. Die Messung der Radioaktivität im Zellkulturmedium lässt keine Aussage zu, ob Cholesterol oder Abbauprodukte, wie Oxysterole detektiert werden. Daher wurde die Regulation der Oxysterolbildung durch die LCM in Kooperation mit Dieter Lütjohann (Spezielle Lipiddiagnostik, Universitätsklinikum Bonn) mit Hilfe eines GC-MS-Ansatzes untersucht. Dabei zeigten sich vielversprechende Änderungen in den Oxysterolkonzentrationen, sodass möglicherweise die Diskrepanz zwischen der verminderten ABCA1-Expression und dem unveränderten Cholesterolefflux auf diese Weise zu erklären wäre. Für den endgültigen Nachweis kann der Oxysterolefflux spezifisch gemessen werden. Als Grundlage könnte dabei die Arbeit von HONG *et al.* (2016) dienen.

Außerdem wird die Regulation der Cholesterolhomöostase durch die LCM in THP-1-Makrophagen untersucht und ein Einfluss von α-TOH und α-13'-OH auf die Biosyntheseprodukte, nicht aber auf das zelluläre Gesamtcholesterol gefunden. Die Untersuchungen zur Regulation der verantwortlichen Enzyme stehen noch aus.

Mit der Untersuchung der möglichen Genotoxizität der LCM wird das Forschungsgebiet erstmals in Richtung Kanzerogenese erweitert. Im Rahmen einer Masterarbeit (Nadine Pritsch, Kooperationsprojekt mit Michael Glei (Lehrstuhl für Ernährungstoxikologie, Institut für Ernährungswissenschaften, Friedrich-Schiller-Universität Jena) werden genotoxische Effekte der GA und α-13'-COOH in RAW264.7-Makrophagen bei hohen Konzentrationen (10 µM) und verhältnismäßig kurzen Inkubationszeiten (4 h) gezeigt. Dies geht mit der Regulation von Genen einher, die am ROS-*Handling* (Katalase, Superoxiddismutase), an der Zellzykluskontrolle (p53, p21) oder im zellulären Entgiftungssystem (Glutathion-S-Transferasen) eine Rolle spielen. Weiterführende Experimente könnten die Induktion einer adaptiven Antwort durch die LCM zeigen. Dazu ist z.B. die Expressionsanalyse bzw. die Untersuchung der Translokation von Nrf2 (*nuclear factor* 2) geplant. Für eine Erweiterung des Projektes sind außerdem *In-vivo*-Genotoxizitätsanalysen geplant, welche in Proben aus dem nachfolgend beschriebenen Projekt durchgeführt werden sollen.

7.4.2 Projekte in *In-vivo*-Modellen

Aufbauend auf den Ergebnissen der Manuskripte 1 und 3, den bereits publizierten Daten, wie sie

unter Kapitel 2.4.1 beschrieben wurden und den unveröffentlichten Daten von Maria Wallert und Andreas Koeberle, konzentrieren sich die kommenden *In-vivo*-Studien auf die antiinflammatorischen Eigenschaften der LCM.

Derzeit befindet sich eine Studie an Wistar-Ratten in Vorbereitung, die die Dosis-Wirkungsbeziehung der LCM in der Endotoxämie untersuchen soll (Hauptverantwortlicher: Adrian Press, Universitätsklinikum Jena). Dazu werden die LCM und GA (1 mg/kg Körpergewicht und 10 mg/kg Körpergewicht) jeweils eine Stunde vor der LPS-Applikation (10 mg/kg Körpergewicht) intraperitoneal verabreicht und Blut sowie Organe werden fünf Stunden nach LPS-Gabe entnommen. Dabei soll die Kurzzeitwirkung der Metabolite im Modell der akuten Inflammation untersucht werden. Messparameter werden unter anderem Konzentrationen von Zytokinen (Tumor-Nekrose-Faktor-α, Interleukin-6, Interleukin-10), der Stoffwechselprodukte der Arachidonsäure oder auch der Testsubstanzen selbst im Blut der Tiere sein. In den entnommenen Geweben wird der Grad der inflammatorischen Reaktion erfasst und insgesamt die Wirkung der LCM und GA bewertet.

Während sich die gerade beschriebene Studie mit der akuten Inflammation beschäftigt, sind Untersuchungen zum Einfluss der LCM auf die stille Inflammation geplant. Im Rahmen einer interdisziplinären Forschergruppe (InflammAging, 0045 FGR 2016), die sich aus den Fachbereichen Medizin, Pharmazie und Ernährungswissenschaften zusammensetzt und durch die Thüringer Aufbaubank gefördert wird, werden die LCM und weitere Naturstoffe in biotechnologisch produzierte Nanocellulose eingebracht, um diese als feuchte Wundauflage bei chronischen Wunden anzuwenden. Zahlreiche Arbeitsschritte sind hierbei nötig, um die lipophilen Naturstoffe zunächst in die hydrophile Nanocellulose einzubringen, ihre Freisetzung zu steuern und die biologische Wirksamkeit *in vitro* und *in vivo* zu bestätigen. Bei erfolgreicher Umsetzung der vorgesehenen Konzepte könnten die wissenschaftlichen Erkenntnisse im klinischen Alltag angewendet und damit in die Industrie transferiert werden.

8 Zusammenfassung

Der Metabolismus von Vitamin E ist seit den 1980er Jahren des vergangenen Jahrhunderts bekannt, wobei die ersten Abbauprodukte, die LCM, erst im Jahr 2002 beschrieben wurden (SONTAG & PARKER). Die biologische Funktion dieser LCM ist ein Forschungsfeld, das bislang nur von wenigen Wissenschaftlern bearbeitet wird, obwohl die Erkenntnisse zu einem erneuten Richtungswechsel in der Erforschung des Vitamin E führen könnten.

In der vorliegenden Arbeit wird mit Hilfe von drei Übersichtsarbeiten (Manuskripte 5 bis 7) der Einstieg in die Thematik ermöglicht, indem diese den aktuellen Kenntnisstand zu den LCM zusammenfassen und die Bedeutung der LCM in den Gesamtforschungskontext einordnen. Außerdem werden in den Übersichtsarbeiten Thesen (T1 bis T5) und Forschungsfragen (F1 bis F5) für weiterführende Untersuchungen abgeleitet. Die Originalarbeiten (Manuskripte 1 bis 4) beschreiben die untersuchten biologischen Effekte der LCM im Hinblick auf die Inflammation und die zelluläre Lipidhomöostase, welche beide relevante Vorgänge in der Atherogenese darstellen. Dabei wird der Großteil der genannten Thesen adressiert (T1 bis T3) und die Forschungsfragen werden partiell (F1 bis F3) bzw. nahezu vollständig beantwortet (F4 und F5).

Der Einfluss der LCM auf die zelluläre Lipidhomöostase wird hinsichtlich der Aufnahme (Manuskript 1) und der Speicherung von Lipiden (Manuskript 4) in humanen Makrophagen (THP-1-Makrophagen) untersucht. Die Expression des Oberflächenrezeptors CD36, welcher für die Aufnahme von oxLDL verantwortlich ist, wird durch eine Inkubation mit LCM erhöht. Eine übermäßige intrazelluläre Akkumulation von Lipiden führt allerdings zur Lipotoxizität. Die LCM zeigen in diesem Zusammenhang einen protektiven Einfluss auf die SA-induzierte Lipotoxizität. Das lipidtropfenassoziierte Protein PLIN2 wird durch die LCM auf mRNA- und Proteinebene induziert und die Neutrallipidspeicherung der Zellen steigt. Durch den PLIN2-spezifischen *Knockdown* wird deutlich, dass die LCM zumindest teilweise über eine PLIN2-Expressionssteigerung vor der SA-induzierten Lipotoxizität schützen.

Die Inflammation wird durch eine LPS-Behandlung von murinen RAW264.7-Zellen imitiert. Die LCM können die LPS-abhängige Induktion der untersuchten Enzyme auf mRNA-, Protein- und funktioneller Ebene hemmen (iNos und Cox2; Manuskripte 1 bis 3). Die Detektion von NO, dem Reaktionsprodukt der iNos, ist in Zellkulturüberständen schwierig. Optimierungsstrategien und ein zuverlässiges Protokoll für die spezifischen hier eingesetzten Bedingungen werden in Manuskript 2 zusammengefasst.

Das Manuskript 1 beschreibt zwar auch die biologischen Effekte der LCM, im Mittelpunkt steht aber der Einfluss der Struktur auf die Effektivität der LCM. Dabei zeigt sich deutlich, dass weder die Vorstufen (α- und δ-TOH) noch die Substrukturen (abgebildet durch Pristansäure und α-CEHC) die LCM-Effekte erzielen können. Diese sind beinahe unabhängig von der Substitution des Chromanolringsystems (α- oder δ-Form), hängen aber entscheidend von der Modifizierung der Seitenkette ab (TOH, 13'-OH, 13'-COOH). So ist die Regulationseffzienz der Carboxychromanole in geringster Konzentration am größten. Diese Ergebnisse legen einen distinkten und spezifischen Regulationsmechanismus für die LCM nahe und bestärken ihre Einordnung als neue Klasse regulatorischer Metabolite. Auf der Basis von Manuskript 1 kann die Identifizierung

putativer LCM-Rezeptoren angestrebt werden. Sollte dies gelingen, könnte die Homologie zu den fettlöslichen Vitaminen A und D bekräftigt werden, da auch ihre Wirkung durch ein physiologisches Abbauprodukt vermittelt wird und ein spezifischer Rezeptor identifiziert werden konnte. Eventuell deutet sich hier ein Konzept an, das auf alle fettlöslichen Vitamine übertragen werden kann.

Die Erforschung der LCM stellt derzeit eine Nische in der Vitamin-E-Forschung dar, welche aber neue und relevante Aspekte zum Gesamtwirkbild des Vitamin E beiträgt. Zahlreiche Ansatzpunkte zur Erweiterung und Vertiefung dieses Projektes werden im Rahmen der vorliegenden Arbeit vorgestellt. Das Ziel, die Vitamin-E-Funktion bis zum hundertjährigen Jubiläum im Jahr 2022 vollständig aufzuklären, erscheint in diesem Licht zwar ambitioniert und anspruchsvoll, die Wissenschaft könnte ihm aber mit der Identifizierung putativer LCM-Rezeptoren und der Erklärung der molekularen Wirkmechanismen einen entscheidenden Schritt näherkommen.

9 Summary

The metabolism of vitamin E has been unraveled in the 1980s, but the existence of its LCM, the first metabolites formed during catabolism, has been shown as late as 2002 by SONTAG & PARKER. Since then, only a few scientists investigated the biological effects of the LCM. Nevertheless, unraveling a putative biological function of the metabolites could change the point of view for vitamin E and its modes of action.

This thesis provides a comprehensive introduction to the biology of the LCM, as three reviews are included (Manuscripts 5 to 7). These manuscripts summarize the current knowledge about the LCM, generate working hypotheses (T1 to T5) and ask questions (F1 to F5), which are relevant for the progress in this research area. The original publications (Manuscripts 1 to 4) deal with the biological effects of the LCM. The studies focus on inflammatory processes and the regulation of cellular lipid homeostasis, both of which are relevant in atherogenesis. Here, most of the hypotheses (T1 to T3) are addressed and the questions are answered in part (F1 to F3) or almost completely (F4 and F5).

Several new findings are described in this thesis: (**i**) The LCM regulate the cellular lipid homeostasis in human THP-1 macrophages by modulating the uptake (Manuscript 1) and storage of lipids (Manuscript 4). The expression of CD36, a scavenger receptor for oxLDL, is induced by the LCM, although the LCM inhibit the uptake of oxLDL, likely by impairing its phagocytic uptake. (**ii**) An excessive intracellular accumulation of lipids leads to lipotoxicity. The LCM protect cells from stearic acid-induced lipotoxicity. The expression of the lipid droplet-associated protein PLIN2 is induced at mRNA and protein level and neutral lipid accumulation is increased by the LCM. The protection against lipotoxicity is partially dependent on the LCM-induced PLIN2 regulation, as shown by PLIN2-specific knockdown. (**iii**) The regulation of inflammatory processes was investigated in LPS-stimulated murine macrophages (RAW264.7 macrophages). It was shown that the LPS-stimulated expression of inflammatory enzymes is efficiently blocked at all levels (mRNA, protein and functional level of iNos and Cox2; Manuscripts 1 and 3). (**iv**) The detection of NO in cell culture supernatants is challenging. Manuscript 2 includes optimization strategies and a solid protocol for the conditions used for investigating the effects of the LCM in the setup described here.

Apart from the biological effects of the LCM, Manuscript 1 focuses on their structure-function relationship. It is evident that neither the precursor (α- or δ-TOH) nor the representatives of LCM-substructures (pristanic acid or α-CEHC) are able to induce effects similar to that of the LCM. The studied effects of the LCM are to a large extent independent from the substitution of the chromanol ring system, but highly dependent on the modification of the side-chain. The carboxychromanols are the most potent molecules despite a lower concentration used. This provides good evidence for a distinct and specific mechanism mediated by the LCM. Furthermore, their classification as a new class of regulatory metabolites becomes evident. Following the results presented in Manuscript 1, the identification of a putative receptor, specific for the LCM, should be the aim of future studies. With the success of this project, the similarity to other fat-soluble vitamins, such as vitamins A and D, is supported, since their physiological metabolites are the

effectors of their function and specific receptors have already been identified for these vitamins. This could lead to a generalization of the concept that bio-activation is in general required for fat-soluble vitamins.

The research on the LCM presented here covers only a part of the efforts to unravel the function of vitamin E, but the results of the presented project add new and highly relevant aspects. For consolidation and extension, several experiments are suggested, which add new facets to the function of the LCM. The aim of future work is to unravel the function of the LCM and their contribution to the function of vitamin E, hopefully until 2022, the centenary anniversary of its characterization as a vitamin.

Nachwort

Als das Forschungsgebiet um den Vitamin-E-Metabolismus entstand, wurde dieser bei einem Vitamin-E-Überangebot für den Abbau des überschüssigen Vitamin E verantwortlich gemacht und sollte so ausschließlich vor einer Intoxikation an Vitamin E schützen. Die dabei gebildeten Metabolite wurden als reine Abbauprodukte angesehen. Schon nach wenigen Jahren wurde die biologische Aktivität der SCM beschrieben (WECHTER *et al.* 1996). Mit fortschreitendem Erkenntnisgewinn ändert sich der Blickwinkel nun auch auf die LCM. So liefern zahlreiche Publikationen deutliche Hinweise für die biologische Aktivität der LCM (siehe Manuskripte dieser Arbeit und alle Publikationen, die unter Kapitel 2.4 beschrieben werden). Die Teilnehmer des 2. Internationalen Vitamin-E-Satellitensymposiums formulierten im zugehörigen Konsensusartikel (GALLI *et al.* 2017) daher folgende Hypothese:

"Given that short chain and even more long-chain metabolites (LCMs) of α-tocopherol are bioactive molecules […], the hypothesis that besides the "excess route" there could be an even more important "activation route" of this vitamin has emerged […]."

Möglicherweise führt der Vitamin-E-Metabolismus also nicht nur zur Ausscheidung von überschüssigem Vitamin E, sondern auch zur biologischen Aktivierung von Vitamin E. Homologe Konzepte sind schon seit langem für die anderen fettlöslichen Vitamine bekannt. Interessanterweise wurden spezifische Rezeptoren für die bioaktivierten Metabolite von Vitamin A und Vitamin D bereits vor einiger Zeit identifiziert (EVANS & MANGELSDORF 2014). Auf dieser Basis könnten die Bestrebungen zur Identifizierung des LCM-Rezeptors vielversprechend verlaufen.

Zusammenfassend kann also gesagt werden, dass die Aufklärung der biologischen Wirksamkeit der Vitamin-E-Metabolite zu einem erneuten Wendepunkt in der Erforschung des Vitamin E führen könnte und die Arbeiten der Forschergruppen einen wesentlichen Beitrag zum Verständnis der Vitamin-E-Funktion liefern.

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B Eigenständigkeitserklärung

Hiermit erkläre ich an Eidesstatt, dass

(i) mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist.

(ii) ich die vorliegende Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe und alle benutzen Hilfsmittel, persönliche Mitteilungen und Quellen in der Arbeit angegeben habe.

(iii) mich lediglich die in den Manuskripten angegebenen Personen bei der Auswahl und der Auswertung des Materials sowie bei der Erstellung der Manuskripte unterstützt haben.

(iv) ich nicht die Hilfe eines Promotionsberaters in Anspruch genommen habe.

(v) Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

(vi) ich diese Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe.

(vii) ich weder die gleiche oder eine in wesentlichen Teilen ähnliche, noch eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, September 2017 _____

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C Curriculum vitae

D Publikationen

Originalarbeiten

Schmölz L, Wallert M, Rozzino N, Cignarella A, Galli F, Glei M, Werz O, Koeberle A, Birringer M, Lorkowski S.
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E Danksagung

Meinen herzlichsten Dank möchte ich gern all denen aussprechen, die mich auf dem Entstehungsweg dieser Doktorarbeit begleitet haben.

Prof. Dr. Stefan Lorkowski danke ich für die kompetente Betreuung, umfassende Unterstützung und immerwährende Förderung, die er mir über die gesamte Zeit der Promotion und darüber hinaus zukommen lässt. Ich danke ihm ganz besonders für das Vertrauen, das er in mich setzt. Seine richtungsweisenden Ideen und kritischen Diskussionen bei experimentellen Entscheidungen haben mich jederzeit vorangebracht. Ich danke ihm für den großzügigen Freiraum, der meine fachliche Weiterentwicklung ermöglicht, z.B. durch zahlreiche nationale und internationale Konferenzbesuche, durch die Möglichkeit, für einen Forschungsaufenthalt nach Innsbruck zu gehen oder auch an zahlreichen Kursen der Graduiertenakademie teilzunehmen. Mein Dank gilt ihm außerdem für die angenehme Arbeitsatmosphäre, das unermüdliche Korrekturlesen meiner Manuskripte und die Genauigkeit, die er mir während unserer Zusammenarbeit vermittelt hat.

Prof. Dr. Marc Birringer danke ich für die Initiation des Themas der langkettigen Vitamin-E-Metabolite hier in Jena und für seine langjährige, kompetente Betreuung. Ich bedanke mich bei ihm für die Bereitschaft, mir und dem gesamten Vitamin-E-Projektteam jederzeit mit seiner analytischen und synthesechemischen Expertise zur Seite zu stehen. Er bereichert mit seinem eigenen Blickwinkel regelmäßig unsere Arbeit, unterstützt uns materiell durch die Bereitstellung von *Garcinia kola* und den Metaboliten und motiviert uns ideell mit der Vision, die Vitamin-E-Funktion bis zum 100jährigen Jubiläum aufzuklären. Ich danke Marc für die unkomplizierte Zusammenarbeit und sein entgegengebrachtes Vertrauen.

Mein besonderer Dank gilt Dr. Maria Wallert, die mir schon als Forschungspraktikantin beim thematischen und experimentellen Einstieg in die Welt der Wissenschaft unterstützt hat. Darüber hinaus hat mich Maria während meiner Diplomanden- und Doktorandenzeit immer hervorragend betreut und umfassend unterstützt. Ich danke ihr für das Vertrauen, das sie mir von Anfang an entgegengebracht hat und für ihre unermüdliche Bereitschaft, mir bei Fragen Antworten zu geben und bei Problemen Lösungsansätze zu entwickeln. Ich danke ihr dafür, dass sie mich stets zur Teilnahme an Konferenzen ermuntert hat und dass sie alle Manuskripte und von mir verfassten Arbeiten Korrektur liest. Ich freue mich, dass Maria zu einer guten Freundin geworden ist, die beruflich und auch privat jederzeit ein offenes Ohr für mich hat.

Für ihre tatkräftige Unterstützung während ihrer Master- und Promotionsphase danke ich Stefan Kluge und Martin Schubert. Ich freue mich immer über die sehr gute Zusammenarbeit und die unkomplizierten Absprachen, die mit den beiden möglich sind. Außerdem danke ich ihnen für den Freiraum, den sie mir während der Schreibphase dieser Arbeit geschaffen haben.

Mein Dank gilt auch Prof. Dr. Andreas Ritsch, der mir den Forschungsaufenthalt in Innsbruck ermöglicht hat. Ich danke ihm für die freundliche Aufnahme in die Arbeitsgruppe, die unerschöpfliche Motivation und moralische Unterstützung während der Laborphase. Zudem bin ich ihm für die zahlreichen Lösungsvorschläge und Ideen zur „Rettung" des Cholesteroleffluxes sehr dankbar.

Ich danke der Masterstudentin Nadine Pritsch für die bienenfleißige Durchführung der Comet-Assays und dem Erasmus-Studenten Nicolò Rozzino für die Unterstützung bei der Durchführung einiger Western-Blots für Manuskript 1.

Der Thüringer Aufbaubank danke ich für die finanzielle Förderung und für die Möglichkeit, im Rahmen der wissenschaftlichen Nachwuchsgruppe Erfahrungen und Expertise zu sammeln.

Allen derzeitigen und früheren Mitgliedern der Arbeitsgruppe danke ich für die gegenseitige Unterstützung, den Zusammenhalt und das gute Arbeitsklima. Ich danke Euch für die abwechslungsreichen Pausen und die individuellen Gespräche.

Bei meinen Mitbewohnerinnen Anke Katharina Müller und Anne-Christin Schneider bedanke ich mich für die stets offenen Ohren, das Aufzeigen von anderen Blickwinkeln, die konstruktive Hilfe bei fachlichen Fragestellungen und ihre eigene Meinung. Ich danke Euch für Euer Verständnis und Eure Geduld während der Schreibphase und hoffe, dass auch ich Euch bei Euren vergangenen und kommenden Schreibphasen ebenso unterstützen konnte und kann.

Anna Rohde danke ich für die ruhige Obhut während der Entstehungsphase der Arbeit und für den ausgezeichneten Tipp mit den Kopfhörern. Ich bin Anna für ihre aufmerksame Art, die freundschaftliche Arbeitsatmosphäre und die gemeinsame Zeit fernab des Instituts sehr dankbar.

Ich bedanke mich von Herzen bei meinen Freunden und bei meiner Familie, die in der letzten Zeit häufiger auf mich verzichten mussten. Vielen Dank für Eure Geduld und Euer Verständnis!

Mein besonderer Dank gilt Sybille Schmölz für das unermüdliche und kritische Korrekturlesen sowie für ihre hilfreichen Impulse bei der Ausarbeitung. Ich bin ihr außerdem sehr dankbar für den moralischen Rückhalt und die verordnete Ruhephase während der Entstehungszeit dieser Arbeit. Für die Unterstützung auf meinem Weg und ihren unbeirrbaren Glauben an mich danke ich ihr von Herzen. Danke!

F Anhang

Tabelle A1: Übersicht zu den recherchierten LCM mit Angabe der Methode.

	Analytik[1]		**Probe**[2]	**Metabolite**		
				α-LCM	γ-LCM	δ-LCM
SONTAG & PARKER (2002)	**D**[1] **E**[1] **C**[1]	- FF GC-MS	3 - 10 ml Medium (HepG2)		γ-7′-COOH γ-9′-COOH γ-11′-COOH γ-13′-COOH γ-13′-OH	
YOU *et al.* (2005)	**D** **E** **C**	- FF GC-MS	Medium (A549 und HepG2)	α-Metabolite, keine explizite Angabe der Form	γ-3′-COOH bis γ-13′-COOH γ-13′-OH γ-T3-5′-COOH bis γ-T3-13′-COOH γ-T3-13′-OH	δ-3′-COOH bis δ-13′-COOH δ-13′-OH δ-T3-5′-COOH bis δ-T3-13′-COOH δ-T3-13′-OH
JIANG *et al.* (2007)	**D** **E** **C**	S FF HPLC-FD	Medium (A549)		γ-9′-COOH bis γ-13′-COOH γ-13′-OH γ-9′S-COOH bis γ-13′S-COOH γ-TOH	δ-9′-COOH bis δ-13′-COOH δ-9′S-COOH bis δ-13′S-COOH
	C	LC-MS (ESI (-))	200 µl Rattenplasma	α-TOH	γ-3′-COOH γ-9′S-COOH bis γ-13′S-COOH γ-13′-COOH γ-13′-OH γ-TOH	
	C	LC-MS (ESI (-))	Rattenleber	α-TOH	γ-9′S-COOH γ-13′S-COOH γ-13′-COOH γ-13′-OH γ-TOH	
FREISER & JIANG (2009a)	**D** **E** **C**	S+G FF HPLC-FD	400 µl Medium (A549)		γ-T3-9′S-COOH bis γ-T3-13′S-COOH γ-T3-9′-COOH bis γ-T3-13′-COOH γ-TOH, γ-T3	δ-TOH
	C	LC-MS (ESI (-))	100 µl Rattenplasma	α-TOH	γ-3′-COOH γ-T3-13′-COOH γ-T3-9′S-COOH bis γ-T3-13′S-COOH γ-TOH, γ-T3	

[1] Die Spalte Analytik enthält Angaben zur **D**ekonjugation, **E**xtraktion und **C**hromatographie der Proben.
[2] Die Probenmenge wurde angegeben, sofern die Information in den Veröffentlichungen zur Verfügung stand.

Tabelle A1 (Fortsetzung)

	Analytik[1]		Probe[2]	Metabolite		
				α-LCM	γ-LCM	δ-LCM
FREISER & JIANG (2009b)	D[1] E[1] C[1]	S+G FF HPLC-FD	400 µl Medium (A549)		γ-9'S-COOH bis γ-13'S-COOH γ-9'-COOH bis γ-13'-COOH	
			100 µl Rattenplasma		γ-T3-3'-COOH γ-T3-9'S-COOH bis γ-T3-13'S-COOH γ-T3-9'-COOH bis γ-T3-13'-COOH	
BIRRINGER *et al.* (2010)	D E C	 HPLC-UV	Medium (HepG2)	α-5'-COOH α-13'-COOH α-13'-OH		
MUSTACICH *et al.* (2010)	D E C	- FF LC-MS	Rattenlebermikrosomen	α-13'-OH		
YANG *et al.* (2010)	D E C	- SPE LC-TOF-MS (ESI (+))	1 ml Medium (A549)		γ-9'-COOH γ-11'-COOH γ-13'-COOH γ-13'-OH	
ZHAO *et al.* (2010)	D E C	S+G FF (2S) HPLC-ECD LC-MS (ESI (-))		Messbar: alle Metabolite von α-TOH, γ-TOH, γ-T3, δ-TOH, δ-T3		
			20 µl humanes Serum	Nur ICM, SCM und TOH		
			20 µl humaner Urin	Nur ICM und SCM		
			10 mg humane Fäzes	α-3'-COOH α-5'-COOH α-13'-COOH α-TOH α-T3	γ-3'-COOH bis γ-11'-COOH γ-TOH, γ-T3	δ-3'-COOH bis δ-11'-COOH δ-TOH
JIANG *et al.* (2013)	D E C	- FF LC-MS/MS (ESI)	30 mg Fäzes		γ-3'-COOH bis γ-13'-COOH γ-13'-OH	δ-3'-COOH δ-7'-COOH bis δ-13'-COOH δ-13'-OH
WALLERT *et al.* (2014a)	D E C	S+G FF Q-TOF-LC-MS (ESI (+))	500 µl humanes Serum	α-13'-COOH		
CIFFOLILLI *et al.* (2015)	D E C	- FF HPLC-ECD GC-MS	1 ml Serum	α-13'-OH		

[1] Die Spalte Analytik enthält Angaben zu der **D**ekonjugation, **E**xtraktion und **C**hromatographie der Proben.
[2] Die Probenmenge wurde angegeben, sofern die Information in den Veröffentlichungen zur Verfügung stand.

Tabelle A1 (Fortsetzung)

	Analytik[1]		Probe[2]	Metabolite		
				α-LCM	γ-LCM	δ-LCM
JIANG *et al.* (2015)	**D**[1] **E**[1] **C**[1]	S FF (2S) HPLC-FD	30 - 50 µl fötales bovines Serum versetzt mit Metaboliten	α-3'-COOH α-5'-COOH	γ-3'-COOH γ-TOH	δ-13'-COOH δ-T3-13'-COOH δ-TOH
	D **E** **C**	S FF (2S) LC/MS/MS (ESI (-))	Medium (A549) für Etablierung der Methode	Messbar: alle Metabolite von α-TOH, γ-TOH, γ-T3, δ-TOH, δ-T3		
			30 mg murine Fäzes	α-13'-COOH α-13'-OH	γ-3'-COOH bis γ-13'-COOH	δ-5'-COOH bis δ-13'-COOH
			30 - 50 µl murines Plasma	α-3'-COOH	γ-3'-COOH γ-9'S-COOH γ-11'S-COOH γ-13'S-COOH γ-13'-COOH	δ-7'-COOH δ-9'S-COOH δ-11'S-COOH δ-13'S-COOH δ-13'-COOH
TORQUATO *et al.* (2016b)	**D** **E** **C**	k.A. FF LC-MS/MS (APCI)	1 ml humanes Serum	α-13'-COOH α-13'-OH		
GIUSEPPONI *et al.* (2017)	**D** **E** **C**	S+G FF LC-MS/MS (ESI (+))	500 µl humanes Plasma oder Serum	α-3'-COOH α-13'-COOH α-13'-OH	γ-3'-COOH	
			100 µl humanes Plasma oder Serum	α-TOH	γ-TOH	
Manuskript 9	**D** **E** **C**	S+G SPE LC-MS/MS/MS	100 µl Plasma	α-3'-COOH α-5'-COOH α-13'-COOH α-13'-OH α-TOH		
	D **E** **C**	S+G SPE HPLC-FD	100 µl Zellsuspension 250 µl Überstand	α-13'-COOH α-13'-OH α-TOH		

[1] Die Spalte Analytik enthält Angaben zu der **D**ekonjugation, **E**xtraktion und **C**hromatographie der Proben.
[2] Die Probenmenge wurde angegeben, sofern die Information in den Veröffentlichungen zur Verfügung stand.
Verwendete Abkürzungen: 2S: Extraktionsverfahren in zwei Schritten: Extraktion der polaren und unpolaren Metabolite aus der gleichen Probe, aber mit unterschiedlichen Lösungsmittelgemischen; α-13'-COOH: α-13'-Carboxychromanol (analog für die anderen Formen und Kettenlängen); α-13'-OH: α-13'-Hydroxychromanol (analog für die anderen Formen); γ-13'-T3-COOH: γ-13'-Carboxychromanol des Tocotrienols (analog für die anderen Formen und Kettenlängen); γ-13'S-COOH: γ-13'-Carboxychromanolsulfat (analog für die anderen Formen und Kettenlängen); APCI: *atmospheric pressure chemical ionization*; ECD: *electron capture detector*; ESI (+) oder ESI (-): Elektronensprayionisation im positiven oder negativen Modus; FD: Fluoreszenzdetektion; FF: Flüssig-Flüssig-Extraktion; GC: Gaschromatographie; HPLC: *high pressure liquid chromatography*; ICM: *intermediate-chain metabolite*; k.A.: keine Angabe des verwendeten Enzyms; LCM: *long-chain metabolite*; LC: *liquid chromatography*; MS: Massenspektrometrie; S: Sulfatase; SCM: *short-chain metabolite*; S+G: Sulfatase und Glucuronidase; SPE: Festphasenextraktion (*solid phase extraction*); TOF: *time of flight*; TOH: Tocopherol; T3: Tocotrienol; UV: Detektor für Ultraviolettstrahlung.